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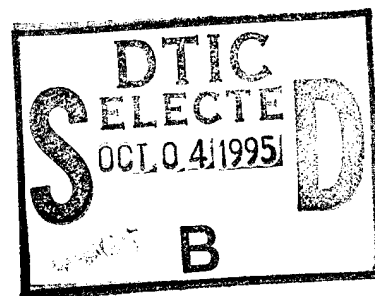
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Pretreatment Dose in Monkeys Challenged with 5 x LD50
Soman and Treated with Atropine/2-PAM"

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ABSTRACT

This task was conducted to determine the minimum dose of pyridostigmine (PYR), and the associated level of erythrocyte acetylcholinesterase inhibition (AChE-I), that provides protection from 5 X 48-hr GD LD₅₀ of untreated monkeys. Monkeys were injected im with GD and treated with 0.4 mg atropine (ATR) free base and 25.7 mg pralidoxime (2-PAM) per kg BW. Phase I experiments were conducted to determine the 48-hr GD LD₅₀ in untreated monkeys and the protective ratio (PR) provided by ATR/2-PAM. Historic and current data were pooled to obtain an overall 48-hr GD LD₅₀ of 6.5 µg/kg. The estimated PR for ATR/2-PAM was 3.2, significantly higher than the 1.7 PR determined in a previous task (85-18). Phase II was performed to determine the im PYR dose which would produce a mean peak (C_{max}) AChE-I of 23 percent and the time (t_{max}) at which the peak occurs. The PYR dose predicted was 24 µg/kg. An im PYR dose of 8.4 µg/kg produced a C_{max} of 9.4 percent. The mean t_{max}, averaged over all PYR dose groups, was 42 min.

In Phase III, survival was measured in 34 animals tested at four PYR doses (0, 4, 8.4, or 24 µg/kg) administered im 45 min prior 32.5 µg/kg GD. None of four monkeys given no PYR survived for 48 hr; 8 of 10 given 4, 9 of 10 given 8.4, and 7 of 10 given 24 µg/kg PYR survived for 48 hr. Probit models failed to adequately describe the relationships between PYR dose or AChE-I with survival. Probabilities of survival were not significantly different among the three PYR doses, but were significantly greater than that for animals not given PYR. Average AChE-I levels among the PYR dose groups were significantly different ($p < 0.0001$) and demonstrated an increasing dose-response relationship. Phase IV was designed to determine an intragastric (ig) PYR dose that produced AChE-I similar to that produced by an effective im PYR dose. An ig PYR dose of 50 µg/kg produced a t_{max} between 120 and 210 min and a AChE-I C_{max} in the range of 5.5-13.5 percent. An ig dose of 40 µg/kg was estimated to result in 5-10 percent AChE-I at 150 min. Eight of 10 monkeys given 40 µg/kg PYR ig 150 min prior to 32.5 µg/kg GD, and treated with ATR/2-PAM, survived for 48 hr.

Phase V evaluated the effect of adding 0.1 mg/kg diazepam (DZM) to treatment regimens. GD dose-lethality responses were studied for three treatment groups: PYR/ATR/2-PAM; PYR/ATR/2-PAM/DZM; and ATR/2-PAM/DZM. The 48-hr GD LD₅₀ for animals treated with ATR/2-PAM/DZM was substantially less than that for monkeys treated with ATR/2-PAM in Phase I. An ATR/2-PAM treatment group was added to Phase V. Probit dose-response models were fitted to the data to estimate the GD dose-lethality relations. The conclusions drawn were: 1) the 48-hr GD LD₅₀s for Phase V animals treated with ATR/2-PAM or ATR/2-PAM/DZM were not statistically different; 2) the 48-hr GD LD₅₀ for Phase I animals treated with ATR/2-PAM was statistically greater than those for animals treated with ATR/2-PAM or ATR/2-PAM/DZM in Phase V; 3) the 48-hr GD LD₅₀s for Phase V PYR-pretreated animals were statistically greater than those estimated for Phase V animals not pretreated with PYR; 4) the 48 hr GD LD₅₀ for animals treated with PYR/ATR/2-PAM was statistically greater than that for animals given PYR/ATR/2-PAM/DZM; the estimated PRs for all PYR-pretreated animals were greater than 14; although the addition of DZM to the treatment regimen reduced the efficacy of PYR/ATR/2-PAM in preventing lethality (PR 27.8), the PR for PYR/ATR/2-PAM/DZM was estimated to be 14.5.

To avoid handling the larger and more aggressive animals in Phase I, monkeys were dosed while restrained within their cages, and ATR/2-PAM injected in the same limb as the GD. In Phase V, animals were removed from their cages, placed on restraint platforms, transported to a chemical fume hood, and injected with GD and treated prior to being returned to their cages. Because these different procedures may have contributed to the difference in 48-hr GD LD₅₀s, 5 animals were injected, while restrained within their cages, at 20.5 µg/kg GD, the Phase I LD₅₀ for ATR/2-PAM animals. Results of Phases I and V were incompatible. This suggests the procedures used contributed to differences in GD LD₅₀s estimated for ATR/2-PAM animals of Phases I and V, although the significance is based on the survival of just one of the five animals.

GOOD LABORATORY PRACTICES COMPLIANCE STATEMENT

In the preparation of a pyridostigmine (Mestinon®) aliquot for nuclear magnetic resonance testing, hydrolysis of the sample occurred and a second aliquot was prepared and analyzed. The raw data file from the first analysis was overwritten and thereby lost. To the best of my knowledge, all other aspects of this study were conducted in compliance with the U.S. Food and Drug Administration's Good Laboratory Practices regulations (21 CFR Part 58).



Carl T. Olson, D.V.M., Ph.D.
Study Director

8/31/95
Date

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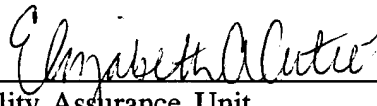
This study was inspected by the Quality Assurance Unit and reports were submitted to the Study Director and management as follows:

Phase <u>Inspected</u>	Date <u>Inspected</u>	Date Reported to Study <u>Director</u>	Date of Report to <u>Management</u>
Dilution/Aliquotting of XGD & 2-PAM	2-10-93	3-1-93	3-1-93
NMR Analysis	2-15-93	3-1-93	3-1-93
Analysis/ID Confirmation of Atropine	2-15-93	3-1-93	3-1-93
ID/Purity of Soman	2-15-93	3-1-93	3-1-93
IR Analysis of 2-PAM	2-16-93	3-1-93	3-1-93
HPLC Identity Confirmation	2-16-93	8-31-93	8-31-93
Sedation, Hair Clipping, Body Weights, Skin Marking, Blood Collection	2-22-93	3-1-93	3-1-93
Loading/Weighing of Syringes, IM Dosing, Observations, Post-Dosing Weights	2-23-93	3-1-93	3-1-93
Post-Pyridostigmine Dosing Blood Analysis	3-2-93	3-31-93	3-31-93
Pyridostigmine Dilution, Body Weights, IV Catheterization, Chairing, Pre-Dose & +5 Minute Blood Collection, IM Dosing	3-2-93	3-31-93	3-31-93
Characterization of Pyridostigmine Bromide Standard	3-4-93	3-31-93	3-31-93
Necropsy, Perfusion, Tissue Harvest	3-9-93	3-9-93	3-9-93
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Dose Calculation, Syringe Loading/Weighing, Oral Dosing, 135 & 145 Minute Blood Collection, COBAS Analysis, Observations	8-10-93	8-31-93	8-31-93
Perfusion, Necropsy	8-13-93	8-31-93	8-31-93

QUALITY ASSURANCE STATEMENT
(Continued)

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<u>Phase Inspected</u>	<u>Date Inspected</u>	<u>Date Reported to Study Director</u>	<u>Date of Report to Management</u>
Pyridostigmine Dose Preparation, Syringe Loading/Weights, Restraint, Blood Collection, Pyridostigmine/GD Dosing, Observations, COBAS Analysis	9-21-93	10-1-93	10-1-93
Hair Clipping, Skin Marking, Blood Collection, GD Aliquotting, Anesthetization	9-23-93	10-1-93	10-1-93
Data/Study File Audits	2-23-93	2-23-93	2-25-93
	6-24-93	6-24-93	7-12-93
	8-12-93	9-20-93	9-23-93
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	1-10-94	1-10-94	1-12-94
	2-15-94	2-15-94	3-2-94
	12-2-94	12-2-94	12-15-94
Audit Draft Final Report	12-22-94	12-22-94	1-16-95
	1-16-95	1-16-95	2-1-95
Audit Final Report	8-28-95	8-28-95	8-30-95


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ODE Inc 4-1-95

8 9-1-95
 Date

EXECUTIVE SUMMARY

MREF Task 92-30 was conducted for U.S. Army Medical Research and Materiel Command (USAMRMC) to determine the minimum effective dose (MED) of pyridostigmine (PYR), and the associated level of erythrocyte acetylcholinesterase inhibition (RBC AChE-I), for protection from 5 X GD LD₅₀ (5 times the soman [GD] dose lethal within 48 hr to 50 percent of challenged, untreated monkeys). For this study, the MED was defined as the minimum dose of PYR which provides an estimated 95 percent survival rate in monkeys injected with 5 X GD LD₅₀ and treated with 0.4 mg atropine (ATR) free base and 25.7 mg pralidoxime chloride (2-PAM) per kg of body weight. The task was conducted in phases, using results of previous phases to assist in the selection of doses for succeeding phases and to reduce the number of animals required.

Phase I experiments were conducted to determine the 48-hr GD LD₅₀ value for untreated monkeys and the protective ratio provided monkeys treated with ATR/2-PAM. Due to body weights in excess of 9 kg for some monkeys in the population allocated for this study, heavier and more aggressive animals were used in Phase I and were injected while restrained in "squeeze-back" cages rather than the usual practice of placing monkeys on restraint boards. Historic and current data were pooled to obtain an overall 48-hr GD LD₅₀ of 6.5 $\mu\text{g/kg}$ when administered intramuscularly (i.m.) in untreated monkeys. The estimated protective ratio (PR) for ATR/2-PAM (i.m.) in an earlier study (Task 85-18) was 1.7 with 95 percent confidence limits of 1.4 to 2.0. In this study, the estimated PR for ATR/2-PAM was 3.2 with a 95 percent confidence interval of 2.4 to 4.1. The PR of ATR/2-PAM in this phase of Task 92-30 was significantly higher than that determined in Task 85-18.

Phase II was performed to determine the i.m. PYR dose which would produce a mean peak 23 percent AChE-I, and to determine the time at which the peak occurs. The PYR dose predicted to produce a peak AChE-I of 23 percent was computed from fitted probit regression equations. The PYR dose producing a peak AChE-I (C_{max}) of 23 percent, based on smoothed AChE-I values, was 24.2 $\mu\text{g/kg}$. An i.m. PYR dose of 8.4 $\mu\text{g/kg}$, a dose approximately 0.45 log units less than that producing 23 percent AChE-I, produced a

smoothed Cmax of 9.4 percent. The overall mean of observed empiric time to Cmax (tmax), averaged over all PYR dose groups, was 42.3 min.

Dose-response experiments with varying doses of i.m. PYR were conducted in Phase III to determine the PYR dose-lethality relationship for monkeys injected with 5 X the 48-hr GD LD₅₀ at the time of predicted maximum PYR-induced AChE-I. Forty-eight hr survival was measured in 34 animals tested at four PYR doses (0, 4.0, 8.4, or 24.0 µg/kg) administered i.m. 45 min prior to a GD dose of 32.5 µg/kg. None of 4 monkeys given no PYR survived for 48 hr; 8 of 10 given 4 µg/kg PYR, 9 of 10 given 8.4 µg/kg PYR, and 7 of 10 given 24 µg/kg PYR survived for 48 hr. Probit models failed to adequately describe the relationships between PYR dose or percent AChE-I with survival. Therefore, contingency table analyses were performed on survival rates categorized by PYR dose. The results indicated that the probability of survival of monkeys at each PYR dose was significantly greater than that observed for the control (0 PYR) group. Probability of survival was not significantly different among the three PYR dose groups. A one-way analysis of variance was performed to compare average AChE-I levels among the PYR dose groups. Differences between groups were highly significant ($p < 0.0001$) and demonstrated a statistically significant increasing PYR dose-AChE-I relationship.

The first portion of Phase IV was performed to determine an intragastric (i.g.) PYR dose that produced a RBC AChE-I level similar to that produced by an i.m. PYR dose effective against a 5 X GD LD₅₀ challenge. Statistical modeling of data for monkeys given an i.g. PYR dose of 50 µg/kg showed a smoothed tmax occurring between 120 and 210 min and a smoothed Cmax in the range of 5.5 - 13.5 percent. It was estimated that an i.g. dose of approximately 40 µg/kg would result in 5-10 percent AChE-I at 150 min following PYR dosing. The second part of Phase IV was designed to determine whether an i.g. PYR dose which resulted in RBC AChE-I similar to that produced by an effective i.m. dose would provide equivalent protection from a 5 X GD LD₅₀ challenge. Eight of ten monkeys given an i.g. PYR dose of 40 µg/kg 150 min prior to a GD injection of 32.5 µg/kg, and treated with ATR/2-PAM 1 min after GD, survived for 48 hr.

The major purpose of Phase V was to evaluate the effect of adding 0.1 mg/kg diazepam (DZM) to treatment regimens. GD dose-lethality responses were studied for three treatment groups: PYR/ATR/2-PAM; PYR/ATR/2-PAM/DZM; and ATR/2-PAM/DZM. Initial results indicated that the 48-hr GD LD₅₀ for animals treated with ATR/2-PAM/DZM was substantially less than that for monkeys treated with ATR/2-PAM in Phase I. To examine this apparent difference in efficacy between animals treated with ATR/2-PAM and animals treated with ATR/2-PAM/DZM, the protocol was amended to include an ATR/2-PAM treatment group in Phase V. Probit dose-response models were fitted to the data to estimate the GD dose-lethality relations for each group of animals. The conclusions drawn from the LD₅₀ ratio comparisons were:

1. The 48-hr GD LD₅₀ for Phase V animals treated with ATR/2-PAM or ATR/2-PAM/DZM are not statistically different.
2. The 48-hr GD LD₅₀ for Phase I animals treated with ATR/2-PAM is statistically greater than those estimated for animals treated with ATR/2-PAM or ATR/2-PAM/DZM in Phase V.
3. The 48-hr GD LD₅₀s for Phase V PYR-pretreated animals are statistically greater than those estimated for Phase V animals not pretreated with PYR.
4. The 48 hr GD LD₅₀ for animals treated with PYR/ATR/2-PAM is statistically greater than that estimated for animals treated with PYR/ATR/2-PAM/DZM. The estimated PRs for all PYR-pretreated animals were greater than 14. Although the addition of diazepam to the treatment regimen may have reduced the efficacy of PYR/ATR/2-PAM in preventing lethality (PR of 27.8), the PR for PYR/ATR/2-PAM/DZM was estimated to be 14.5.

In Phase I, to avoid handling of the larger and more aggressive animals, monkeys were dosed while restrained within their cages, and ATR and 2-PAM were injected in the same limb as the GD. In Phase V, animals were removed from their cages, placed on restraint platforms, transported to a chemical fume hood, and injected with GD and treated prior to being returned to their cages. Because these different procedures may have contributed to the difference in 48-hr GD LD₅₀s estimated for animals treated with ATR/2-PAM in Phases I and V, the protocol was amended and five animals were injected, while restrained within their cages, at a fixed GD dose of 20.5 µg/kg, the 48-hr GD LD₅₀

estimated for animals treated with ATR/2-PAM in Phase I. The lethality results in the five animals dosed while restrained in their cages are statistically incompatible with the GD dose-lethality relation estimated for the eight animals in Phase V that were injected with GD and ATR/2-PAM while restrained on platforms. While this result suggests that the procedures used contributed to the difference in 48-hr GD LD₅₀s estimated for ATR/2-PAM treated animals dosed in Phases I and V of this experiment, the significance is based on the survival of just one of the five animals, and therefore must be interpreted with caution.

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TASK 92-30
DETERMINATION OF THE MINIMUM EFFECTIVE PYRIDOSTIGMINE
PRETREATMENT DOSE IN MONKEYS CHALLENGED WITH 5 X LD₅₀ SOMAN
AND TREATED WITH ATROPINE/2-PAM

1.0 INTRODUCTION

Current U.S. Army therapy for countering exposure to the organophosphonate pinacolylmethylphosphonofluoridate (soman; GD) is pretreatment (administration prior to exposure) with the carbamate pyridostigmine bromide (PYR) and treatment following exposure with atropine (ATR) and pralidoxime chloride (2-PAM). The reversible carbamate acetylcholinesterase (AChE) inhibitor prevents the irreversible binding of GD to the AChE molecule. The minimum effective dosage of PYR, however, has not been established. In a previous experiment conducted at Battelle's Medical Research and Evaluation Facility (MREF)⁽¹⁾, rhesus monkeys given 1.2 mg/kg PYR by nasogastric intubation every 8 hr for a total of 6 doses prior to GD, and ATR/2-PAM therapy following a GD challenge, were effectively protected. Pretreatment with PYR, in conjunction with ATR/2-PAM treatment, was shown to provide significantly improved protection from GD-induced lethality than ATR/2-PAM therapy alone. Additional research is necessary to determine the minimum effective PYR dose, the relationship of erythrocyte (RBC) AChE inhibition (AChE-I) to PYR dose, and the effect of adding an anticonvulsant, such as diazepam (DZM), to the treatment regimen.

The primary objective of this task was to determine the minimum effective dose (MED) of PYR, and the associated RBC AChE-I level, for protection from 5 X GD LD₅₀ (5 times the GD dose lethal within 48 hr to 50 percent of challenged, untreated monkeys). For this study, the MED of PYR was defined as the minimum dose of PYR which provides an estimated 95 percent survival rate in monkeys injected with 5 X GD LD₅₀ and treated with 0.4 mg ATR free base and 25.7 mg 2-PAM per kilogram of body weight.

Secondary objectives of the study were to determine:

- a. the relationship between RBC AChE-I and PYR dosage;
- b. the relationship between RBC AChE-I, induced by PYR, and lethality in monkeys injected with 5 X GD LD₅₀ and treated with ATR/2-PAM;

- c. the effect on the severity of GD intoxication of adding 0.1 mg/kg DZM to the ATR/2-PAM therapy; and
- d. treatment efficacy of an intragastric (i.g.) dose of PYR creating AChE-I levels in monkeys comparable to those observed for the intramuscular (i.m.) MED of PYR.

2.0 EXPERIMENTAL DESIGN

2.1 Test Animals

Male rhesus monkeys, *Macaca mulatta*, were specified for this study because there is considerable scientific evidence that the monkey is predictive of GD therapeutic responses in man. Rhesus monkeys of Indian origin were selected because the majority of work in this area has been done with monkeys of Indian origin, and because there is evidence that rhesus monkeys of Chinese origin respond somewhat differently to these study conditions than those of Indian origin.⁽²⁾

One hundred and six monkeys for use in this study were provided by the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) and were received at Battelle on June 16, 1992 although actual experimentation did not begin until February, 1993. Monkeys weighed approximately 4 to more than 9 kg, and were housed individually in stainless-steel cages approximately 24 inches wide, 34 inches high, and 26 inches deep. Room temperatures were maintained, as possible, between 65 and 84 degrees F, and relative humidity at 30 to 70 percent. Fluorescent lighting on a light/dark cycle of 12 hr each per day was used. Purina Certified Monkey Chow[®] biscuits were fed twice daily and supplemented with locally purchased fresh fruit or vegetables twice weekly. Chemical analyses of certified feed are on file at Battelle. Water was supplied *ad libitum* through automatic watering systems. Water is analyzed for chemical impurities and potability annually. No contaminants that would interfere with the results of this study are known to be present in the food or water.

All animals arrived with tattoos so that positive identification could be maintained. Monkeys were quarantined for more than six weeks, during which time they were examined by a Battelle veterinarian and blood samples taken for hematology and serum chemistry analyses. Fecal samples were taken and examined for gastrointestinal parasites and three tuberculin tests were performed at approximately 2 week intervals during quarantine.

Two monkeys (79V, 6ZU) failed to thrive, had chronic diarrhea, and experienced loss of body weight despite supportive efforts. Both monkeys died within a month of arrival, prior to release from quarantine. Necropsies failed to reveal explanations for these deaths. A third monkey (H306) demonstrated muscular wasting of the lower extremities and, following consultation with a U.S. Army laboratory animal veterinarian, was euthanatized on June 30, 1993. Necropsy revealed healed fracture sites in the lower lumbar vertebrae. Bony deformities probably were responsible for spinal cord and/or spinal nerve damage which led to decreased mobility and muscle wasting.

One hundred and three monkeys were accepted for use in this study. Sixteen monkeys were used in Phase I, 10 were used in Phase II pharmacodynamic studies and were used again in later phases, 34 were used in Phase III, 10 in Phase IV, and 43 in Phase V, as amended.

2.2 Materials and Methods

Experiments were conducted in a stage-wise fashion to minimize the number of animals used to achieve statistically valid results. Discomfort and injury of animals was limited to that which was unavoidable in the conduct of scientifically valuable research. Anesthetics, analgesics, or tranquilizers could not be used for the relief of pain or anxiety at the time of GD injection in these studies because such compounds could interfere with the physiological effects of the GD and therapy compounds. External stimuli and manipulation were minimized to decrease any associated anxiety. Ten days after GD injection, surviving monkeys were sedated with ketamine hydrochloride, anesthetized with pentobarbital sodium, and perfused with neutral buffered formalin, and tissue samples were harvested. Animals dying on study were necropsied and tissue samples taken. If, in the opinion of the Study

Veterinarian or Study Director, conditions existed such that continuation on study would be inhumane, monkeys were anesthetized and necropsied prior to the tenth day. No monkeys were to be sacrificed within 48 hr of GD injection, however, since previous experience with this compound suggests that no reliable method exists to predict survivability during the acute phase of intoxication.

2.2.1 Chemistry

Atropine (ATR) in citrate buffer was donated, at the request of USAMRICD, by Survival Technology, Inc. Verification of identity by nuclear magnetic resonance (NMR) and determination of concentration by high pressure liquid chromatography (HPLC; MREF Method No. 2/Chemistry; see Appendix B) was accomplished at Battelle. Analysis established a 2.54 mg ATR free base/mL concentration. Small aliquots were prepared for use on dosing days and stored at approximately 5 ($\pm$ 3) C. Atropine solution aliquots were passed through sterile 0.2 micron filters (Acrodisc®, Gelman Sciences, Ann Arbor, MI) prior to use in Phase III and subsequent phases. At completion of the study, reanalysis of the atropine solution yielded a comparable concentration of 2.46 mg ATR free base/mL.

2-PAM powder (WR-016411BD, BK96362) and analysis data were provided by the Walter Reed Army Institute of Research (WRAIR). A solution for dosing this compound was prepared by weighing 75 g of the 2-PAM in a 250-mL volumetric flask and adding distilled water. Analysis by HPLC (MREF Method No. 1/Chemistry; see Appendix B) determined the concentration to be 318 mg/mL. Approximately five to six-mL aliquots of this 2-PAM solution were prepared and stored at approximately 5 ($\pm$ 3) C until the day of use. Reanalysis at the completion of the study yielded a concentration of 304 mg/mL.

PYR powder (WR-250710BD, BM03894) and analysis data were provided by WRAIR. Prew weighed 25-mg amounts were provided in 30 amber-glass, screw-top vials along with additional bottles of 100 mg or 1,000 mg quantities. Vials were stored in a desiccator at approximately -15 ($\pm$ 10) C. Solutions with nominal concentrations of 0.25 or

1.0 mg PYR/mL were prepared on each day of i.m. dosing using the provided pre-weighed 25-mg samples and deionized water. Analysis of the first dosing solution showed a 0.29 mg/mL rather than a 0.25 mg/mL concentration, and the second dosing solution was 0.26 mg/mL. Thereafter, when dosing solutions were prepared, the vial was weighed prior to and after removing the PYR to estimate the actual amount of PYR in the vial, and dosing was accomplished based on the concentration estimated by weight loss. All dosing solutions were within approximately ± 5 percent of that estimated following this procedure except for two days in Phase V in which the analyzed concentrations were approximately 93 and 112 percent of that expected.

A one-pint bottle of PYR syrup (Mestinon®, Hoffmann-LaRoche) with a labeled concentration of 12 mg/mL was purchased from ICN Pharmaceuticals, Inc. (Irvine, CA). Identity was confirmed by NMR and concentration analysis by HPLC (MREF Method No. 10/Chemistry; see Appendix B) yielded 12.6 mg pyridostigmine bromide/mL. Mestinon syrup was diluted 1:9 with deionized water to form a solution with a nominal PYR concentration of 1.26 mg/mL. This was aliquoted in approximately 3-mL volumes into amber vials and stored at room temperature. Reanalysis of the Mestinon syrup at the completion of the study yielded a concentration of 12.8 mg pyridostigmine bromide/mL.

Diazepam (DZM; Valium®, Steris Laboratories, Inc.) with a labeled concentration of 5 mg/mL was purchased from W.A. Butler Co. (Columbus, OH) and aliquoted in approximately 3-mL volumes into amber vials and stored in a locked medical safe at room temperature. HPLC analysis (MREF Method No. 11/Chemistry; see Appendix B) confirmed identity and determined concentration to be 5.18 mg/mL. A repeat analysis following completion of the study determined concentration to be 5.02 mg/mL.

Soman was provided by USAMRICD. GD dosing solutions were prepared by diluting neat GD stock with physiological saline solution. Various GD concentrations, ranging from approximately 0.15 to 1.7 mg/mL, depending upon the needs in different phases of the experiment, were prepared, aliquoted into septum-capped vials, and stored at approximately -80 (± 10) C. After preparation, concentrations of the solutions were determined by gas chromatography (GC), and dosing volumes were calculated using these

analytical chemistry results. After each day that dosing was accomplished, the GD dosing solution was analyzed to confirm concentration. Chemical analyses generally demonstrated a concentration within ± 10 percent of that predicted and were never less than 90 percent of that concentration. Dilutions of several dosing solutions were prepared and used to inject mice to confirm agent potency biologically.

2.2.2 Study Phases

Experiments were performed in phases, using results of previous phases to assist in the selection of doses for succeeding phases and to reduce the number of animals required. A short description of the phases follows.

Phase I: Initial tests to confirm GD LD₅₀s for untreated and ATR/2-PAM treated animal.

Phase II: Estimation of i.m. PYR dose producing 23 percent mean peak RBC AChE-I.

Phase III: Estimation of i.m. PYR MED for a 5 x 48-hr GD LD₅₀.

Phase IV: Pharmacodynamics of i.g. PYR and efficacy studies of i.g. PYR pretreatment.

Phase V: Effect of diazepam on efficacy of PYR pretreatment.

Phase I was performed, using a maximum of 10 animals in each of the two groups, to provide estimates of the 48-hr GD LD₅₀s in monkeys given no therapy and in monkeys given 0.4 mg ATR free base and 25.7 mg 2-PAM/kg body weight. This was accomplished using a modified up-and-down experimental design⁽³⁾, challenging only a few monkeys per day and increasing or decreasing the GD doses based on results obtained to date, and assuming a GD dose-lethality response slope consistent with results from earlier Battelle experiments. After 4 or more monkeys were challenged with GD, the estimated GD LD₅₀ for untreated monkeys was statistically compared to the Battelle historic LD₅₀ in rhesus monkeys. If the difference between the two LD₅₀s was determined to be statistically

insignificant at the 5 percent level, the Battelle historic LD₅₀ data would be updated with these additional results and the updated value accepted for this population of animals. An estimate of the GD LD₅₀ in monkeys given ATR and 2-PAM therapy sequentially starting 1 min following injection of GD was accomplished in a similar up-and-down type of experiment. If the estimated 48-hr protective ratio (PR; LD₅₀ of treated animals divided by the LD₅₀ of untreated animals) fell within 1.4 - 1.9 after 4 or more monkeys had been challenged and treated, this phase of the study would end.

Approximately 24 hr prior to challenge, animals were sedated with ketamine hydrochloride (Vetalar®, Fort Dodge, Ft. Dodge, IA) and weighed, injection sites were clipped of hair and circumscribed with color-coded permanent markers, and a blood sample was taken (heparinized 2-mL Vacutainer® with 22-gauge, 1-inch needle, Becton-Dickinson, Rutherford, NJ) for AChE activity determination.⁽⁴⁾ Body weights taken at this time were used to calculate volumes of challenge agent and therapy compounds. Syringes used for dosing were calibrated Hamilton Company (Reno, NV) gas-tight microliter syringes of the smallest compatible capacities to obtain maximum accuracy in delivered volumes. Twenty-five gauge, 5/8-inch needles (Becton-Dickinson) were used for all i.m. injections. After the GD dosing solution was thawed in a chemical fume hood, individual, labeled syringes were loaded with the calculated volumes prior to the start of dosing, weighed, and placed on ice until used. After dosing was accomplished, syringes were weighed again to determine the weight losses and calculate the volumes delivered. Pre- and post-injection weighings of syringes were also accomplished with those used for dosing ATR and 2-PAM. Soman was injected i.m. in the calf in the region of the gastrocnemius muscle, and ATR and 2-PAM were injected sequentially i.m. in the anterior lateral aspect of the thigh in the region of the vastus lateralis muscle. Decontamination of the skin surrounding the i.m. GD dosing site was accomplished first with swabs soaked in ethanol and then swabs soaked in 5 percent sodium hypochlorite. This sequence was repeated and then the site rinsed twice with water-soaked swabs. Monkeys were monitored continuously for a minimum of 2 hr following GD injection and at decreasing frequency thereafter for 10 days. The incidence of signs of GD

intoxication, including tremors, convulsions, salivation/bronchial discharge, prostration, and death, was recorded.

Some animals in the population of monkeys allocated for this study weighed more than 9 kg, and heavier and more aggressive monkeys were used in Phase I and were injected while restrained in "squeeze-back" cages rather than the usual practice of hand-catching and placing monkeys on restraint platforms. Because of difficulty in adequately restraining both hind limbs for injections, GD, and ATR/2-PAM were injected into the same limb although at distant sites. This also is different from procedures used in earlier studies. Monkeys dying on study were necropsied and tissue samples harvested and preserved in formalin. Animals surviving for 10 days were sedated with ketamine and then deeply anesthetized with pentobarbital sodium. This was followed by a buffered formalin perfusion, necropsy and tissue sampling.

Phase II was performed to estimate the i.m. PYR dose required to produce a 23 percent peak RBC AChE-I. This was the mean level of RBC AChE-I measured in monkeys 4 hr following the 6th and final 1.2 mg/kg i.g. dose of PYR given every 8 hr which proved efficacious in mitigating the effects of GD intoxication in monkeys of an earlier study.⁽¹⁾ Initially, two monkeys were injected i.m. in the region of the vastus lateralis muscle with 10.5 μ g/kg of PYR and blood samples were taken at approximately 5 min before and 5, 10, 20, 30, 45, 60, and 90 min after PYR injection. An additional blood sample often was taken approximately 10 min prior to PYR dosing to provide a baseline AChE level in case problems arose with the analysis of the sample taken 5 min before PYR dosing. Results of the analyses of the -10 min blood samples were not necessary and were not used. Blood was separated into cells and plasma by centrifugation and the packed RBCs analyzed for AChE activity using an automated centrifugal chemical analyzer (COBAS FARA®, Roche Diagnostics, Branchburg, NJ).⁽⁴⁾ Based upon the peak RBC AChE-I observed during this time period, PYR doses were altered in subsequent studies to obtain an estimate of the PYR dose which causes a peak AChE-I level approximating 23 percent. Animals studied in this phase were used again after a minimum one week washout period, and were also used in later phases of the study. Once a PYR dose estimated to produce approximately 23 percent

RBC AChE-I was determined, all 10 monkeys of this phase were given a similar dose and AChE-I analyzed as a function of time.

Once the approximate i.m. PYR dose required to produce a mean peak 23 percent AChE-I was estimated, two of the same monkeys used earlier in this phase were injected with a PYR dose approximately 0.45 log units lower to determine whether AChE-I at this lower PYR dose was significantly greater than zero and if time to peak AChE-I differed from that with the higher dose. If significant AChE-I was obtained, the remaining eight animals used in the original study of this phase were to be injected also with this PYR dosage and blood samples collected. If significant AChE-I was not obtained with the lower dose, the PYR dose would be increased to 0.30 log units less than the dose required to produce 23 percent AChE-I, and the study repeated.

To perform this phase of the study, each monkey was acclimated to a restraint chair prior to experimentation. An indwelling catheter (18-gauge or 21-gauge, 4½-inch Intrafusor®, Abbott, Chicago, IL) was placed in a saphenous vein, and the monkey restrained in a chair. The monkey was injected with PYR in the quadriceps muscle of the leg without the catheter. To maintain patency of catheters, approximately 0.7 mL of a heparinized saline solution (approximately 30 units heparin/mL; heparin from Elkins-Sinn, Cherry Hill, NJ; 0.9 percent sodium chloride solution from Baxter, Deerfield, IL) was injected into catheters following blood collections. At the time of blood sampling, the heparin block was drawn off and discarded, and approximately 1-1½ mL of blood was drawn into a 3-mL heparinized syringe (Becton-Dickinson). In an effort to maintain patency, at times a new heparin solution block was instilled in the catheter between +60 and +90 min blood draws. If catheter patency was lost during experimentation, blood samples were obtained by femoral or saphenous sticks using a vacutainer® or a heparinized syringe and needle. The blood was processed⁽⁴⁾ to determine AChE activity, and AChE-I was calculated by dividing activity levels at different times by the -5 min AChE activity determination and subtracting this value from 1. If the time of blood sampling was delayed, the actual time the sample was drawn was used to model AChE-I kinetics.

In Phase III, stage-wise experiments were conducted with varying doses of i.m. PYR to determine the PYR dose-GD induced lethality response slope. Also to be determined were the PYR ED₅₀ (PYR dose effective in preventing lethality in 50 percent of animals challenged with 5 X 48-hr GD LD₅₀) and the PYR MED for monkeys injected with 5 X 48-hr GD LD₅₀ at the time of predicted maximum PYR-induced AChE-I and treated with 0.4 mg/kg ATR free base and 25.7 mg/kg 2-PAM starting 1 min following GD injection. Most of the preparations and procedures previously described for Phase I were used in Phase III. Major exceptions were the consistent injection of GD in the right calf while the monkey was confined on a restraint board and placed within a chemical fume hood, and ATR/2-PAM injections consistently being given in the lateral quadriceps muscle of the left leg.

Early stages of the experiment focused on high and low doses of PYR to ensure that lethality was observed in monkeys given low doses of PYR and that monkeys given sufficiently high doses of PYR survived. Initially, using two monkeys at each PYR dose, monkeys were to be treated with the i.m. PYR dose predicted to produce 23 percent AChE-I, and at a dose approximately 0.45 log units less if significant AChE-I was produced at this PYR dose. In all subsequent stages, doses of PYR used would depend upon the 48-hr survival results obtained in previous stages.

Approximately 24 hr prior to GD challenge, study monkeys were sedated with ketamine and weighed, injection sites were clipped of hair and marked, and blood samples were taken to establish baseline AChE activities. On the day of study, monkeys were injected with PYR, preferably in the quadriceps muscles of the right leg but often in the left, while restrained in "squeeze-back" cages. A few min before the time of predicted peak AChE-I, each monkey was hand-caught and placed on a slotted, V-shaped platform and limbs restrained. A blood sample was taken approximately 5 min prior to GD injection to determine the AChE-I. Monkeys were then brought to a chemical fume hood approved for the use of highly hazardous materials. GD was injected, with the monkey within the hood, in the right leg in the posterior tibial area in the region of the gastrocnemius muscle. The site of GD injection was decontaminated and the monkey removed from the hood. Atropine and 2-PAM, in succession, were injected i.m. at separate sites 2 to 3 cm distant from each

other in the quadriceps femoris muscle of the left leg starting one min after the injection of GD. To obtain maximum accuracy in the measurement of delivered doses, syringes used for dosing were microliter syringes of the smallest compatible capacities. Syringes were filled to no more than 95 percent of labeled total volume. In some cases, more than one atropine injection was necessary due to the size of the animal and the subsequently large dosing volume (> 1.2 mL) required. Each labeled syringe was loaded with the calculated volume of GD dosing solution prior to the start of dosing, weighed and placed on ice until used. After dosing was accomplished, syringes were weighed again to determine the weight losses and calculate the volumes delivered. Pre- and post-injection weighings of syringes were also accomplished with those used for dosing PYR, ATR, and 2-PAM. Monkeys were monitored continuously for the first two hr following GD injection and at a decreasing frequency thereafter for 10 days.

If all monkeys given a PYR dose predicted to produce a mean 23 percent peak AChE-I level did not survive for 48 hr following GD challenge and ATR/2-PAM therapy, the USAMRICD point of contact (POC) and the U.S. Army Contracting Officer's Representative (COR) were to be notified before further research was conducted. If the slope of the PYR dose-GD induced lethality response was low (< 1) such that reasonable estimation of a MED became difficult, further research would cease, the POC and the COR notified, and possible modifications of the experimental design discussed. Otherwise, this phase of experimentation would end when the standard error of the estimated i.m. PYR ED_{50} was less than 20 percent or after a maximum of 50 monkeys had been challenged.

Phase IV was designed to determine efficacy of an i.g. PYR (Mestinon®) pretreatment at a dose that produced AChE-I levels similar to that produced by the i.m. PYR MED for a 5 X 48-hr GD LD_{50} challenge. Initially, two monkeys previously acclimated to chair restraint were given a dose of PYR syrup by intragastric intubation and AChE-I measured over time. Each of the monkeys was fasted overnight, and then restrained on a board. Baseline blood samples were obtained by femoral venipuncture approximately 10 and 5 min prior to dosing with PYR. An oral feeding tube (15 in, No. 8 French; Ethox Corp., Buffalo, NY) was passed through a nostril and inserted to the level of the stomach, a few mL

of water injected to assure proper placement, and the measured dose of PYR contained in a microliter syringe inserted in the tube and flushed with approximately 5 mL of water. An intravenous catheter was placed in a saphenous vein and taped in place, as in Phase II. Monkeys were placed in restraint chairs and blood samples taken at approximately 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, and 180 min following PYR administration and analyzed for AChE activity. In general, after taking the 180 min samples, monkeys were returned to their cages. Additional blood samples were obtained from some monkeys while they were still in restraint chairs if analysis results did not indicate clearly that a AChE-I zenith had been reached or to obtain a better estimate of the pharmacodynamic kinetics. Results were used to obtain an estimation of the maximum AChE-I attained, the time to maximum AChE-I, the rate of change in AChE-I, and the variability between animals. The approximately -5 min blood sample was used as the baseline AChE activity determination.

Additional monkeys were treated similarly using various doses of i.g. PYR to estimate the PYR dose-AChE-I response. Monkeys used in these studies were sometimes dosed again after a washout period, and were also used in later phases of the experiment.

Following completion of the analysis of the data obtained, a total of 10 monkeys, using two at a time, were fasted overnight, and then had two baseline blood samples taken approximately 10 and 5 min prior to PYR dosing. Monkeys were dosed with PYR syrup i.g., and when AChE-I levels were predicted to approximate that seen with an effective i.m. PYR dose, challenged with 5 X GD LD₅₀ and treated i.m. sequentially with 0.4 mg ATR free base and 25.7 mg 2-PAM/kg body weight starting at 1 min following the i.m. injection of GD. Monkeys given PYR were restrained a few minutes before the predicted time of maximum AChE-I, blood samples were taken approximately 15 and 5 min prior to GD challenge, and the monkeys injected with GD and treated with ATR/2-PAM. AChE-I calculations were based on the AChE activity measured in the blood samples taken -5 min prior to dosing with PYR; -10 min blood samples were taken in case difficulties in analysis of the -5 min blood sample occurred. Procedures used for GD dosing and ATR/2-PAM therapy were similar to those described for Phase III. Monkeys were monitored continuously for a minimum of 2 hr following GD challenge and at decreasing frequency thereafter for

10 days. Incidences of signs of GD intoxication were recorded. The 48-hr survival results for monkeys given an i.g. dose of PYR were compared to the survival rate of monkeys given an i.m. dose of PYR which produced a similar AChE-I at the time of GD injection to determine whether a statistically significant difference existed.

Phase V was designed to determine the effect on the PYR MED of adding diazepam to the ATR/2-PAM treatment regimen. The efficacy of various treatments in preventing GD-induced death was evaluated by estimating the 48-hr GD LD₅₀ in monkeys receiving a given treatment. This was accomplished using a modified up-and-down experimental design, challenging a few monkeys per day for each treatment and increasing or decreasing the GD dose based on results obtained. Treatments evaluated were: 1) 0.4 mg/kg ATR/25.7 mg/kg 2-PAM/0.1 mg/kg DZM, with all treatments given i.m. sequentially starting at 1 min following challenge with various doses of GD; 2) PYR i.m. prior to GD challenge and ATR/2-PAM i.m. as in 1) above; and 3) PYR i.m. prior to GD challenge and ATR/2 PAM/DZM i.m. as in 1) above.

Using chemical restraint (ketamine), all monkeys were weighed approximately 24 hr prior to GD dosing, injection sites were clipped of hair and marked, and blood samples were taken by femoral venipuncture to establish a baseline AChE activity. These weights were used to calculate the doses of GD and treatment compounds. Another blood sample was taken just prior to GD injection to determine AChE-I level. Monkeys receiving the PYR pretreatment were injected in the right lateral quadriceps muscles while restrained in a "squeeze-back" cage. A few minutes prior to the time of scheduled GD injection, each monkey was hand-caught and placed on a restraint platform, and a blood sample obtained by femoral venipuncture approximately 5 min prior to GD challenge. Each monkey was brought to a chemical fume hood, injected with GD in the right calf, the injection site decontaminated, the animal removed from the hood and then treated with ATR/2-PAM or ATR/2-PAM/DZM i.m. at separate sites sequentially in the left quadriceps starting 1 min following the GD injection. Some monkeys required more than one injection of atropine.

Monkeys were returned to their cages and monitored continuously for a minimum of 6 hr and at decreasing frequency thereafter for 10 days. Signs of GD intoxication were

recorded. The number of animals used in this phase was to depend upon the results of 48-hr survival. If the standard error of the estimated 48-hr GD LD₅₀ following dosing of a minimum of five animals for any treatment regimen was less than 10 percent, testing of that treatment would cease. No more than 10 monkeys would be used for estimating the 48-hr GD LD₅₀ for any treatment.

2.2.3 Pathology

Animals surviving for 10 days, or monkeys after 48 hr following GD injection for which it was deemed inhumane to continue on study, were sedated with ketamine and then deeply anesthetized with pentobarbital sodium. This was followed by a buffered formalin perfusion. A complete necropsy, with tissue harvesting, of all animals that died on study or animals that were anesthetized and perfused during the study or at the end of the 10 day holding period was accomplished, and animal remains were cremated. Tissue samples taken included: brain; spinal cord; dorsal root ganglia; peripheral nerves (brachial plexus, median [main trunk in upper arm and one muscular branch in lower arm where it enervates flexor carpi radialis muscle], phrenic [attached to diaphragm], and sciatic to include the main trunk, common peroneal branch overlying the lateral wing of the gastrocnemius muscle, and tibial branch just caudal to the bifurcation); eye; heart with aorta; kidneys; liver; gall bladder; lung; spleen; pancreas; stomach; duodenum; jejunum; ileum; cecum; colon; abdominal skin; mesenteric lymph node; thyroids with parathyroids; testis; bone marrow of rib and femur; thymus; skeletal muscles (muscles innervated by collected peripheral nerves, including samples of flexor carpi radialis, biceps brachii, diaphragm, intercostal, anterior tibialis, biceps femoris, and lateral head of the gastrocnemius); trachea; esophagus; parotid salivary gland; urinary bladder; pituitary gland; and adrenal glands. Tissues removed and preserved in formalin were sent to USAMRICD for histopathologic evaluation.

2.2.4 Statistical Methods

Statistical analyses were conducted on three types of experimental data: dose-response data, pharmacodynamic data, and clinical signs data. All of the statistical programs were written in SAS (Statistical Analysis Systems, version 6.04, Cary, NC).

2.2.4.1 Dose-Response Data

Throughout the course of the study, experimental data were generated to assess four dose-response relationships:

- 1) GD dose vs. lethality,
- 2) PYR dose vs. AChE-I,
- 3) PYR dose vs. GD-induced lethality, and
- 4) AChE-I vs. GD-induced lethality.

Probit dose-response models⁽⁵⁾ were fitted to the data using Proc NLIN in SAS. The following model was fitted to the GD dose-lethality data for each group of treated animals:

$$p = \Phi[a + b * \log_{10}(\text{GD } \mu\text{g/kg})],$$

where, p is the probability of lethality, and Φ is the standard normal probit transformation. The estimated parameters were used to calculate the 48-hr GD LD₅₀s and associated 95 percent confidence intervals. Confidence intervals for LD₅₀s were calculated using Fieller's Theorem.⁽⁵⁾ LD₅₀s were compared among treated groups of animals using PRs and LD₅₀ ratios. The PR was defined for each group of treated animals as the ratio of the LD₅₀ for treated animals to the LD₅₀ for untreated animals. Confidence intervals for PRs and LD₅₀ ratios were calculated using the delta method.⁽⁶⁾

The probit dose-response regression model fitted to the PYR dose-AChE-I data was

$$C_{\max} = \Phi[a + b * \log_{10}(\text{PYR } \mu\text{g/kg})],$$

where, $C_{\max}$ is the maximum AChE-I attained during the experiment. The fitted parameters were used to estimate the PYR dose required to induce specified peak AChE-I levels. In addition, analyses of variance (ANOVA)⁽⁷⁾ were carried out to compare the mean peak percent AChE-I between PYR dose groups. Tukey's⁽⁷⁾ and Dunnett's⁽⁷⁾ multiple comparison tests were conducted to determine significant differences (5 percent level) in peak AChE-I between specific pairs of PYR doses. The ANOVA and multiple comparisons procedures were accomplished using Proc GLM in SAS.

Contingency table analyses were conducted to assess PYR dose-GD induced lethality data. Fisher's Exact Tests⁽⁷⁾ were performed to assess the homogeneity of survival between two PYR dose groups, and chi-square tests⁽⁷⁾ were employed to assess survival rates among three or more PYR dose groups. Contingency table analyses were completed using Proc Freq in SAS.

2.2.4.2 Pharmacodynamic Data

For each animal and PYR dose, $C_{\max}$ was defined as the maximum AChE-I value attained during the time period of the study, and $t_{\max}$ was defined as the corresponding timepoint. Empiric values of $C_{\max}$ and $t_{\max}$ were calculated from the AChE-I time-course data for each experiment.

For each animal and PYR dose, the following quadratic regression model was used to smooth the AChE-I data :

$$\text{Percent AChE-I} = b_0 + b_1 * \text{time} + b_2 * \text{time}^2 ,$$

where, b_0 = intercept, b_1 = linear coefficient of time, and b_2 = quadratic coefficient of time. The regression models were fitted using Proc Reg in SAS. The estimated coefficients for b_0 , b_1 , and b_2 were then used to calculate the model smoothed values of Cmax and tmax. Smoothed Cmax was defined to be the maximum AChE-I value attained by the fitted quadratic regression model during the duration of the experiment, and smoothed tmax was defined to be the corresponding timepoint. If the fitted regression equation predicted that the smoothed tmax occurred prior to the first sampling timepoint, the first sampling timepoint was assumed as the smoothed tmax and used in the regression equation to calculate the smoothed Cmax. On the other hand, if the fitted regression equation was a strictly increasing function, the last sampling timepoint was reported as the smoothed tmax and was used in the regression equation to calculate the smoothed Cmax. The probit dose-response regression model, as described, was fitted to empiric and smoothed Cmax values to estimate the PYR dose necessary to produce specified peak levels of AChE-I.

2.2.4.3 Clinical Signs Data

The following responses were recorded, as applicable, for each animal: appeared normal, tremors in upper body, tremors in lower body, convulsions, salivation (excessive salivation or bronchial discharge), miosis, mydriasis, prostration, and death. The occurrence of each sign in each of the monitored time intervals was noted and stored in a Paradox® (Version 4.0, Borland International, Scotts Valley, CA) database. In creating the computerized database, tremors noted in either upper or lower body were combined into one tremor response. This resulted in a total of eight clinical signs for statistical analyses.

For each clinical sign the following three endpoints were calculated:

- (a) Time to onset (time between GD injection and first observation of sign).
- (b) Duration of sign during the first 2 hr after GD injection.
- (c) Duration of sign during the first 6 hr after GD injection.

Lethality was also analyzed using the methods described in Section 2.2.4.1. In that section, lethality was defined as death within 48 hr following GD injection. To maintain consistency between the analysis of lethality using the methods described in Section 2.2.4.1 and those discussed below, incidence of lethality was based on 48 hr results and time to death, if greater than 48 hr, was treated as right censored. Three types of statistical analysis were conducted for each clinical sign:

- (1) Fisher's Exact Test was conducted to compare the incidence of the clinical sign between two groups. This analysis was performed using Proc Freq in SAS.
- (2) Nonparametric ANOVA⁽⁸⁾ (Wilcoxon test) was used to compare the clinical signs among two or more groups. For this analysis, the time to onset was set equal to missing if the sign was not noted for an animal. This analysis was performed using Proc NPAR1WAY in SAS.
- (3) Parametric ANOVA appropriate for censored data⁽⁹⁾ was performed using the SAS procedure PROC LIFEREG. The ANOVA model included an intercept for each group and was fitted with and without a covariate (slope) for the logarithm of GD dose. Times to onset were assumed to be log-normally distributed, and durations within 2 hr and 6 hr were assumed to be normally distributed. In this analysis, time to onset was treated as right censored at the time of death (minimum) or 240 hr (maximum), if the sign was not observed. Time to death was treated as right censored at 48 hr. Also, the durations of signs were defined as right censored if the sign occurred and the animal died within the specified time interval (2 or 6 hr). For instance, if convulsions started at 15 min following GD injection and continued until the animal died at 1 hr after injection, the duration of the convulsions was defined to be right censored at 45 min. Mean times to onset and durations, based on ANOVAs, were predicted for each clinical sign. Predicted mean times based on ANOVA models that included a covariate for log GD dose were computed at the 48-hr GD LD₅₀ for each group.

3.0 RESULTS

3.1 Statistical Analyses

3.1.1 Phase I

Phase I studies to determine the 48-hr GD LD₅₀ value for untreated monkeys and the PR provided monkeys treated with ATR/2-PAM were accomplished between February 23

and March 9, 1993. A total of six untreated monkeys and ten monkeys treated with ATR/2 PAM were dosed with GD in a modified up-down manner. Table D-1 in Appendix D is a listing of animals, body weights, RBC AChE activity levels prior to dosing, GD doses, and 48-hr survival status.

A probit dose-response model in log GD dose was fitted to the 48-hr lethality data from the 6 untreated monkeys and the data from 19 untreated monkeys from Tasks 89-08⁽¹⁰⁾ and 89-12⁽¹¹⁾. This model assumed that the GD dose-lethality curves for the two groups (the historical data and the current Task 92-30 data) had a common slope but separate intercepts. Table 1 presents the slope, estimated 48-hr GD LD₅₀s, and 95 percent confidence limits based on this model. Analogous results based on separate models fitted to the two groups are presented in Table D-2 in Appendix D.

TABLE 1. COMPARISON OF RESULTS OF PROBIT ANALYSES ON UNTREATED MONKEY DATA

Treatment Group	N	Slope (S.E.)	Slope 95% Confidence Limits	LD ₅₀ (S.E.) (μ g/kg)	LD ₅₀ 95% Confidence Limits
89-08, 89-12 (Untreated)	19	13.9 (5.2)	[3.7, 24.0]	6.7 (0.03)	[5.8, 8.5]
92-30 (Untreated)	6	13.9 (5.2)	[3.7, 24.0]	5.7 (0.05)	[4.1, 7.6]

A chi-square test was performed to determine whether the LD₅₀s estimated for the historic and current untreated groups were statistically different. If the two LD₅₀s are statistically equivalent, then the data can be combined to more precisely estimate the LD₅₀ for the present group of animals. The resulting value of the chi-square statistic was 1.71 with 1 degree of freedom; this was less than the 5 percent significance cutoff value of 3.84. Since the chi-square test was not significant, the historic and current data were combined into a single group of untreated animals and a probit dose-response model was fitted to the combined 48-hr lethality data. The slope of the GD dose-lethality curve was estimated to be 12.1 with 95 percent confidence limits of 3.2 and 20.9, and the 48-hr GD LD₅₀ was

estimated to be 6.5 $\mu\text{g/kg}$ with a 95 percent confidence interval of 5.6 to 8.0 $\mu\text{g/kg}$. The data from untreated animals of Tasks 89-08, 89-12, and 92-30 were pooled for the remaining statistical analyses.

A probit log GD dose-lethality response model with one slope and four intercepts was fitted to the data from four groups of animals: (a) Task 85-18 untreated animals,⁽¹⁾ (b) Task 85-18 ATR/2-PAM treated monkeys,⁽¹⁾ (c) pooled Tasks 89-08⁽¹⁰⁾, 89-12⁽¹¹⁾, and 92-30 untreated monkeys, and (d) Task 92-30 ATR/2-PAM treated monkeys. Figure 1 displays the GD dose-lethality curves estimated for the four groups of animals. Table 2 presents the slope, LD₅₀s, PRs, and 95 percent confidence limits. Analogous results based on separate models fitted to each of the four groups of animals are presented in Table D-3, and are statistically equivalent to those displayed in Table 2. The LD₅₀s for the Task 85-18 untreated and ATR/2-PAM-treated groups are 15.1 $\mu\text{g/kg}$ and 25.0 $\mu\text{g/kg}$, respectively. The LD₅₀s for the Task 92-30 untreated and ATR/2-PAM-treated groups are 6.5 $\mu\text{g/kg}$ and 20.5 $\mu\text{g/kg}$, respectively. For Task 85-18, the estimated PR was 1.7 with 95 percent confidence limits of 1.4 and 2.0. For Task 92-30, the estimated PR is 3.2 with a 95 percent confidence interval of 2.4 to 4.1. The ratio of the Task 92-30 PR divided by the Task 85-18 PR is 1.9 with a 95 percent confidence interval of 1.4 to 2.6. This confidence interval does not contain 1.0, demonstrating that the PR for Task 92-30 is significantly higher than that estimated for Task 85-18. As shown in Figure 1, the GD dose-lethality curves estimated for the untreated and ATR/2-PAM groups from Task 92-30 are further apart than those estimated for Task 85-18. This reflects the greater PR estimated for ATR/2-PAM in Task 92-30 than in Task 85-18.

FIGURE 1. PREDICTED PERCENT LETHALITY VERSUS GD DOSE FOR UNTREATED AND ATROPINE/2-PAM TREATED MONKEYS

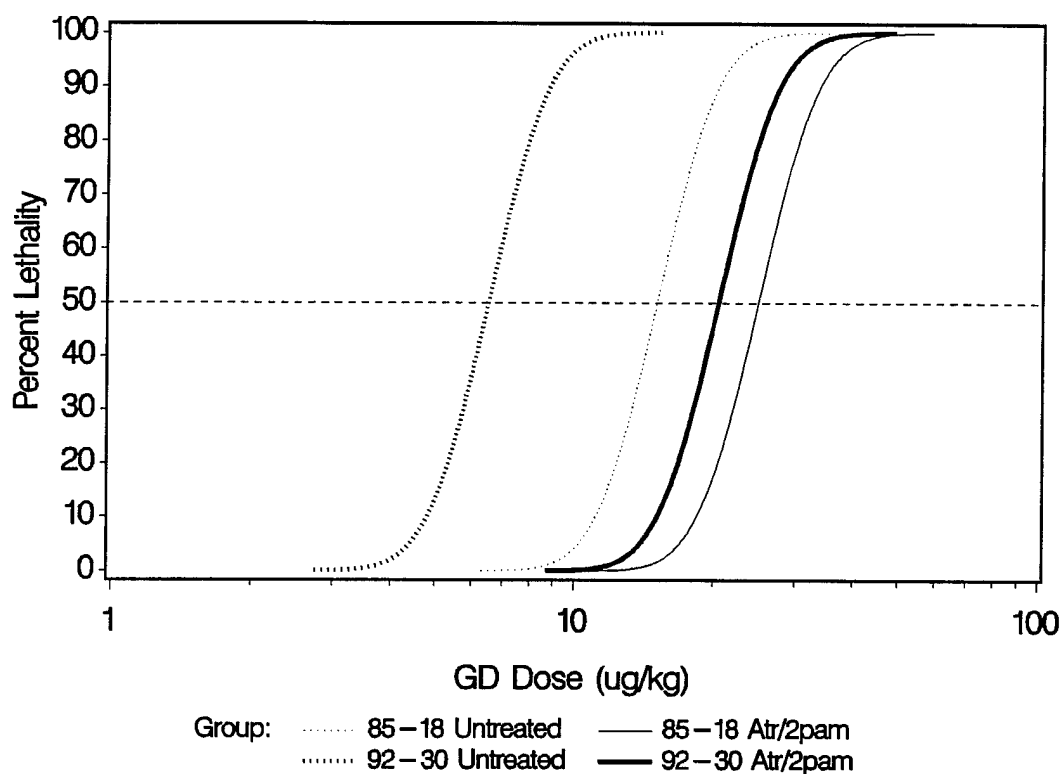


TABLE 2. SUMMARY OF RESULTS OF PROBIT ANALYSES ON TWO UNTREATED AND TWO ATROPINE/2-PAM TREATED GROUPS USING COMMON-SLOPE (9.76) MODEL

Treatment Group	N	LD ₅₀ (S.E.)	LD ₅₀ 95% Confidence Limits	Protective Ratio	Protective Ratio 95% Confidence Limits
85-18 Untreated	36	15.1 (0.03)	[13.1, 17.1]		
85-18 ATR/2PAM	28	25.0 (0.03)	[22.1, 28.6]	1.7	[1.4, 2.0]
89-08, 89-12, 92-30 Untreated	25	6.5 (0.03)	[5.6, 7.6]		
92-30 ATR/2PAM	10	20.5 (0.05)	[16.2, 26.2]	3.2	[2.4, 4.1]

3.1.2 Phase II

Experiments were conducted in Phase II between March 2 and March 31, 1993 to estimate the i.m. PYR dose which would produce a mean peak 23 percent AChE-I, and the time following PYR dosing at which the peak occurs. The ten animals used in this phase were dosed with PYR in multiple experiments with a minimum 1-week washout period between dosings. Altogether a total of 28 experiments were conducted. Initially, two animals were injected with 10.5 $\mu\text{g/kg}$ PYR, and blood samples were taken at approximately -5, 5, 10, 20, 30, 45, 60, and 90 min. Subsequent PYR doses were selected in a stagewise fashion to establish the PYR dose predicted to produce an approximately 23 percent peak AChE-I. Two animals were injected at each of the following target doses: 10.5, 18, 25, 33, and 80 $\mu\text{g/kg}$. Twenty-three percent AChE-I was predicted to occur at a PYR dose in the range of 25 to 27 $\mu\text{g/kg}$. Ten animals were tested at a PYR dose in that range of 25 to 27 $\mu\text{g/kg}$, and the same ten animals were injected again with a PYR dose of 8.4 $\mu\text{g/kg}$.

Table D-4 in Appendix D is a listing, in increasing order of PYR dose, of AChE-I following PYR injection. Two individual time values flagged with asterisks were considered to be outliers and were not used in the following analyses. A quadratic regression model was fitted to the AChE-I data for each animal and PYR dose. Figure 2 displays observed and fitted AChE-I levels for each animal tested at both 8.4 and 25-27 $\mu\text{g/kg}$ i.m. PYR doses. A separate graph is produced for each animal, displaying results for both PYR doses. Mean percent AChE-I levels were computed at each time point for each of the two PYR doses (8.4 and 25-27 $\mu\text{g/kg}$) by averaging the percent AChE-I over all ten animals within a dose group. Quadratic regressions were fitted to the average time course data for the two doses. Figure 3 displays observed and fitted AChE-I levels for the averaged responses for the two dose groups.

FIGURE 2. OBSERVED AND MODELLED PERCENT AChE INHIBITION VERSUS TIME FOR TWO PYR DOSES PLOTTED SEPARATELY FOR EACH ANIMAL

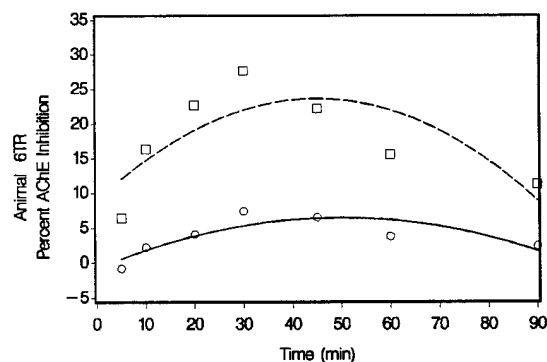
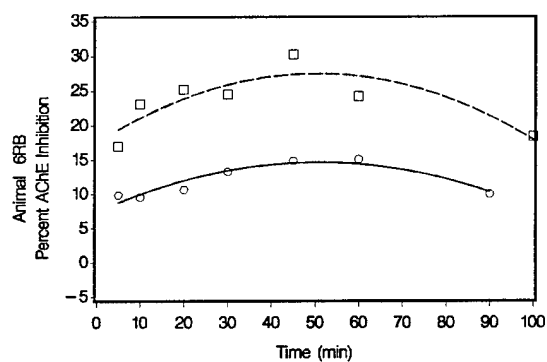
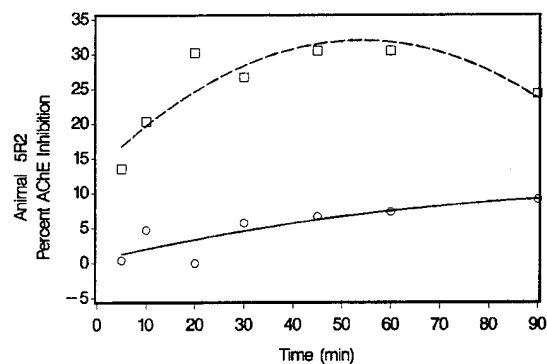


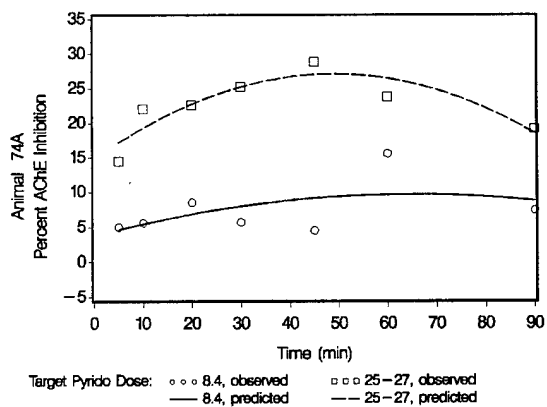
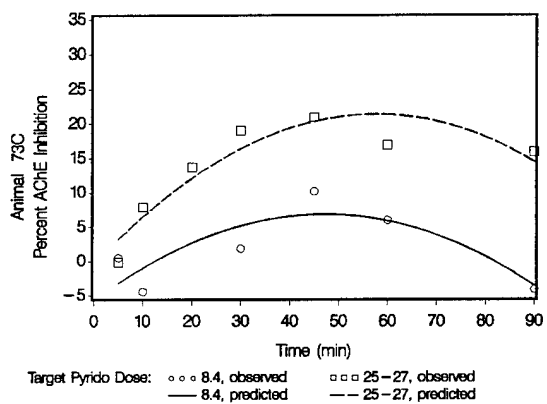
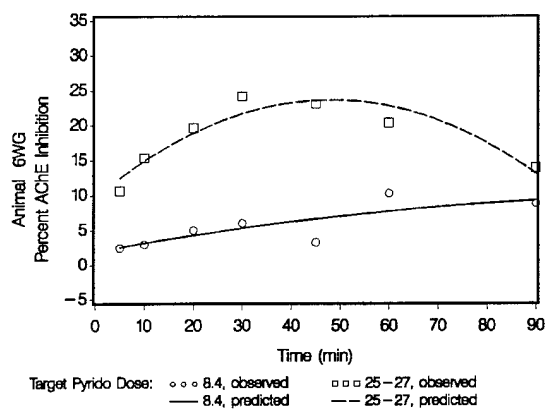
FIGURE 2.
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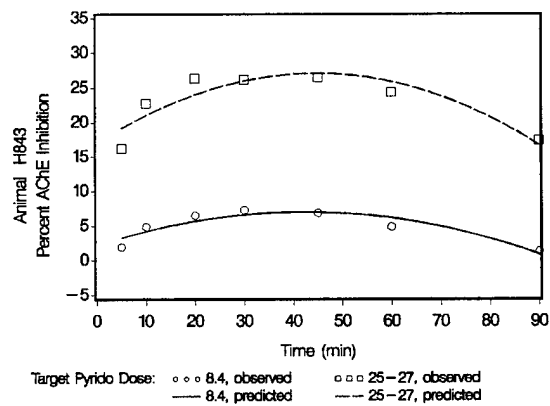
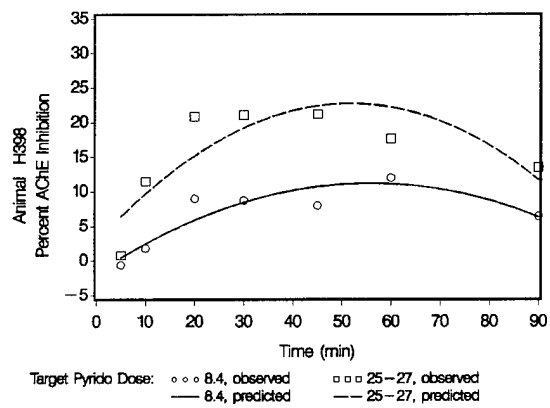
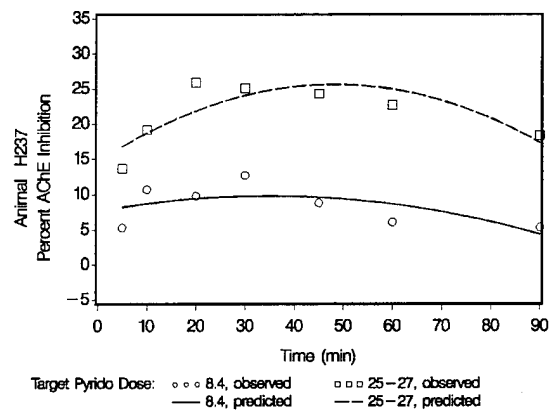
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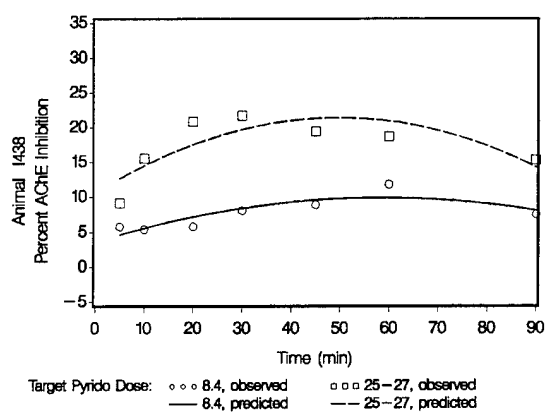
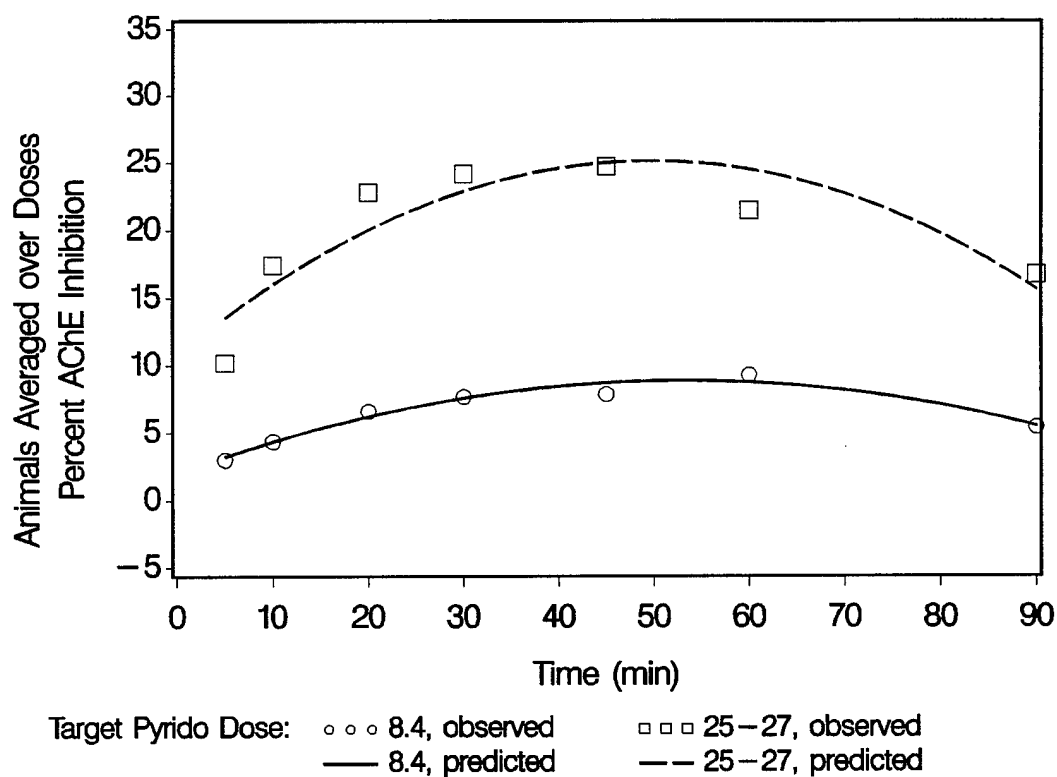


FIGURE 3. PLOT OF OBSERVED AND MODELLED PERCENT AChE INHIBITION VERSUS TIME AVERAGED OVER TEN ANIMALS AT EACH OF TWO PYR DOSES



For each of the 28 experiments, AChE-I time course data were used to estimate the empiric and smoothed C_{max} and t_{max} values. Table D-5 in Appendix D presents these values for each of the 28 experiments. Descriptive statistics for empiric and smoothed C_{max} and t_{max} for the two PYR dose groups are presented in Table 3. One experiment from the 8.4 µg/kg dose group and one from the 25-27 µg/kg dose group were omitted from the descriptive statistics due to outlying values of observed t_{max}.

TABLE 3. DESCRIPTIVE STATISTICS OF EMPIRICAL AND SMOOTHED PARAMETERS FROM QUADRATIC REGRESSION FOR TWO PYRIDOSTIGMINE DOSES

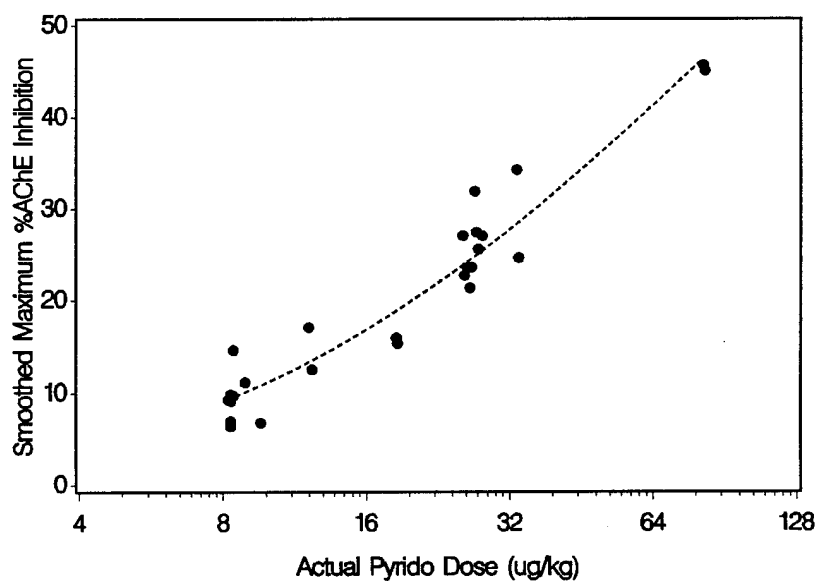
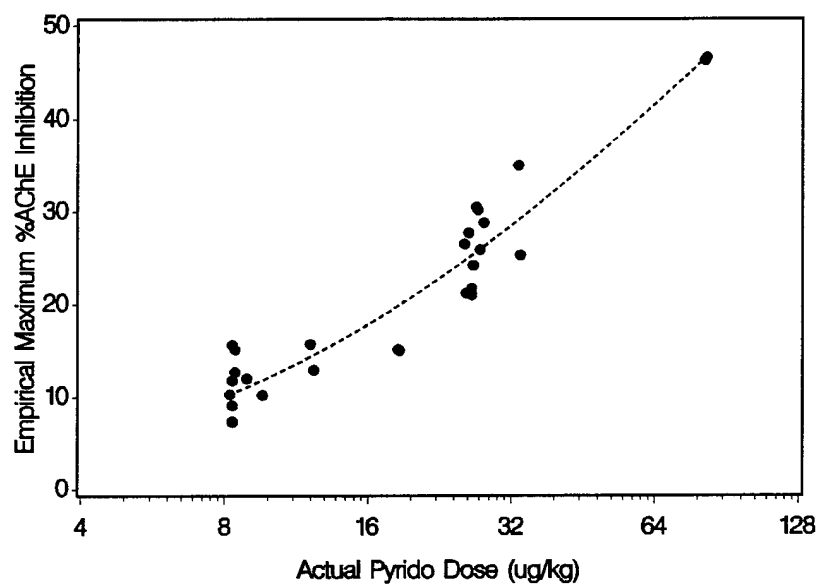
Pyridostigmine Dose (µg/kg)	Parameter	N	Minimum	Maximum	Mean	Std Dev
8.4	Empirical C _{max}	9	7.3	15.6	11.4	2.9
	Smoothed C _{max}	9	6.4	14.7	9.4	2.6
	Empirical t _{max}	9	30.0	60.0	48.3	14.6
	Smoothed t _{max}	9	34.5	129.4	59.2	27.8
25-27	Empirical C _{max}	9	21.0	30.5	25.8	3.8
	Smoothed C _{max}	9	21.4	31.9	25.1	3.5
	Empirical t _{max}	9	30.0	45.0	40.0	7.5
	Smoothed t _{max}	9	44.7	57.6	50.0	4.1

Note: Parameters for 5R2 were omitted from descriptive statistics for 8.4 µg/kg PYR dose.

Parameters for H237 were omitted from descriptive statistics for 25-27 µg/kg PYR dose.

A probit dose-response regression model was fitted to the empiric and smoothed C_{max} values. Slopes of the PYR dose-peak AChE-I curves were significant at the 5 percent level. The predicted PYR dose-peak AChE-I curves, together with the empiric and smoothed C_{max} values, are shown in Figure 4. Parameters of the fitted dose-response models were used to estimate the dose of PYR predicted to produce a peak AChE-I of 23 percent. The PYR dose producing an empiric C_{max} of 23 percent was 22.9 µg/kg with 95 percent confidence limits of 20.9 and 25.0 µg/kg, and the PYR dose producing a smoothed C_{max} of 23 percent was 24.2 µg/kg with a 95 percent confidence interval of 22.4 to 26.1 µg/kg.

FIGURE 4. PLOTS OF EMPIRICAL AND SMOOTHED C_{max} VALUES AS FUNCTIONS OF PYR DOSE WITH MODELLED PROBIT CURVES



The relationship between i.m. PYR dose and tmax was also investigated. There were no recognizable dose-response patterns detected between PYR dose and empiric or smoothed tmax. The mean empiric tmax, averaged over all PYR dose groups, was 42.3 min.

3.1.3 Phase III

Dose-response experiments with varying doses of i.m. PYR were conducted in Phase III between April 6 and June 8, 1993 to determine the PYR dose-lethality relationship for monkeys injected with 5 X 48-hr GD LD₅₀ at the time of predicted maximum PYR-induced AChE-I. The 48-hr GD LD₅₀ of 6.5 µg/kg was obtained from the results using six animals in Phase I pooled with data from 19 animals from previous studies conducted at Battelle's MREF.^(10,11) Therefore, the 5 X GD LD₅₀ dose used for all animals in Phase III was 32.5 µg/kg.

A blood sample was taken the day prior to PYR injection to determine baseline AChE activity level, and another blood sample was obtained approximately 5 min before GD injection to determine percent AChE-I. GD was injected at 45 min following the i.m. PYR injection, the approximate time of predicted maximum AChE-I based on Phase II results. Animals were then treated with 0.4 mg atropine free base and 25.7 mg 2-PAM per kg of body weight starting 1 min following GD injection.

Because of concerns that the slope of the PYR dose-GD induced lethality relationship might prove to be very shallow, the experimental approach for this phase was a mixture of two designs. Initially, experiments would focus on fixed doses of PYR, one predicted to produce 23 percent AChE-I and a lower dose producing significant AChE-I. PYR doses used in subsequent stages were to depend upon survival observed in previous stages, with an adaptive PYR dose allocation being implemented only if permitted by the slope of the PYR dose-GD induced lethality relationship.

During the first week of Phase III, eight animals were tested, four at a PYR dose of 8.4 µg/kg and four at a dose of 24.0 µg/kg. The 8.4 µg/kg i.m. PYR dose produced an

average of approximately 12 percent AChE-I and the 48-hr survival of these 4 monkeys challenged with 5 X 48-hr GD LD₅₀ was 100 percent. The AChE-I values of these eight animals at approximately 40 min following PYR injection were treated as C_{max} values and pooled with the 28 smoothed C_{max} values from Phase II. The pooled data were fitted to a PYR dose-AChE-I C_{max} regression model similar to that used for the data of Phase II alone. The fitted model was then utilized to predict the PYR doses to produce peak AChE-I of 5 and 23 percent. The PYR dose calculated to produce 5 percent peak AChE-I was 4.0 µg/kg, with a 95 percent confidence interval of 3.1 to 5.0 µg/kg. Therefore, a PYR dose of 4.0 µg/kg was selected as the low dose for Phase III studies.

A total of 34 animals were tested at four PYR doses (0, 4.0, 8.4, or 24.0 µg/kg). Table D-6 in Appendix D presents a listing of individual animal body weight, GD dose, PYR dose, baseline AChE activity level, percent AChE-I, and 48-hr result. Table 4 presents summary statistics computed for each PYR dose group, displaying percent survival and means and standard deviations for percent AChE-I. Due to the shallow slope of the PYR dose-GD induced lethality relationship, and the fact that 4 µg/kg PYR provided protection equivalent to that of 24 µg/kg PYR, the U.S. Army COR was consulted and further Phase III experiments were not accomplished.

TABLE 4. SUMMARY STATISTICS FOR PHASE III RESULTS
BY PYRIDOSTIGMINE DOSE

Pyridostigmine Dose (µg/kg)	Number Tested	Number Surviving	Percent Surviving	Mean (S.D.) of % AChE-I
0.0	4	0	0	3.0 (0.5)
4.0	10	8	80	6.9 (2.3)
8.4	10	9	90	12.1 (3.5)
24.0	10	7	70	28.7 (3.3)

3.1.3.1 Statistical Models of Survival versus Pyridostigmine Dose and AChE-I

Survival was analyzed as a function of PYR dose and as a function of percent AChE-I using probit dose-response models. Neither probit slope was significant, and both models failed to adequately describe relationships with survival. An attempt was made to model the probability of survival for animals injected with 5 X 48-hr GD LD₅₀ as a function of both PYR dose and AChE-I using a bivariate probit dose-response model. Again, neither the slope for AChE-I or PYR dose was statistically significant. Furthermore, due to the strong linear correlation between PYR dose and AChE-I, results from the model for some of the PYR doses were nonsensical.

Although a general trend exists between increasing AChE-I and survival, a predictive model between AChE-I and survival could not be developed. Due to the limited amount of information collected at levels of AChE-I less than 5 percent, the data were insufficient to adequately model the relationship between PYR dose and AChE-I with survival. Therefore, the probit models could not be utilized to estimate a minimum effective PYR dose.

Contingency table analyses were performed to confirm that PYR dose administration did significantly alter survival rates. Two types of analyses were performed:

- 1) A chi-square test was conducted to determine if the probability of survival is homogeneous among the three PYR dosed groups (4.0, 8.4, and 24.0 $\mu\text{g/kg}$).
- 2) A Fisher's Exact Test was conducted for each PYR-dosed group to statistically compare the probability of survival between the PYR-dosed group and the control group.

The conclusions drawn from the results of the contingency table analyses are:

- (1) survival rates are significantly greater for each of the PYR-dosed groups relative to the control group, and (2) survival rates are not statistically different among the three PYR-dosed groups.

3.1.3.2 Analyses of Variance of AChE Inhibition Levels by Pyridostigmine Dose

One-way analyses of variance were performed to compare average AChE-I levels among the four groups (control and three PYR doses). As presented in Table 4, the average AChE-I levels corresponding to the 0, 4, 8.4, and 24 $\mu\text{g/kg}$ PYR dose groups are 3.0, 6.9, 12.1, and 28.7 percent, respectively. The results of the analyses of variance were statistically significant ($p < 0.0001$).

Tukey's and Dunnett's multiple comparison tests were also performed to determine significant differences between specific pairs of PYR doses. The results of these tests showed that all pairwise comparisons are significant except for the control group compared with the 4 $\mu\text{g/kg}$ PYR group (3.0 versus 6.9 percent). The 8.4 and 24 $\mu\text{g/kg}$ PYR groups showed significantly greater average percent AChE-I than the control group. The 8.4 $\mu\text{g/kg}$ group had significantly greater AChE-I than the 4.0 $\mu\text{g/kg}$ group, and the 24 $\mu\text{g/kg}$ group had significantly greater AChE-I than the 8.4 $\mu\text{g/kg}$ group. These analyses demonstrated a significant increasing dose-response relationship between PYR dose and percent AChE-I.

To summarize results of Phase III studies, 48-hr survival was measured in 34 animals tested at four PYR doses (0, 4.0, 8.4, and 24.0 $\mu\text{g/kg}$) administered i.m. prior to a GD dose of 32.5 $\mu\text{g/kg}$. The results of the statistical analyses are:

- (1) Probit models were used to attempt to describe survival of individual animals as a function of PYR dose and as a function of percent AChE-I levels. Neither probit slope was significant, and both models failed to adequately describe relationships with survival. Therefore, probit models could not be utilized to estimate a PYR ED_{50} or MED.
- (2) Contingency table analyses were performed on survival rates categorized by PYR dose. The results showed that the probability of survival of monkeys at each PYR dose was significantly greater than that observed for the control group. Probability of survival was not significantly different among the three PYR doses.
- (3) One-way analyses of variance were performed to compare average AChE-I levels among the PYR dose groups. Differences between groups were highly significant ($p < 0.0001$) and demonstrated a statistically significant increasing dose-response relationship.

3.1.4 Phase IV

Phase IV was conducted between June 22 and August 24, 1993.

3.1.4.1 Intragastrically-Administered PYR/AChE-I Pharmacodynamic Studies

Pharmacodynamic studies were performed to measure the effects of intragastrically-administered PYR on AChE-I levels. Baseline blood samples were drawn, monkeys dosed intragastrically with PYR, catheters placed in saphenous veins, and blood samples drawn at approximately 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, and 180 min (and occasionally at 195 and 210 min) after PYR dosing. Experiments were conducted at three i.g. PYR doses: 0, 50, or 125 $\mu\text{g}/\text{kg}$.

Table D-7 in Appendix D presents the data of the pharmacodynamic experiments of Phase IV. A quadratic regression equation was fitted to AChE-I values as a function of time for each experiment. Table D-8 in Appendix D displays the smoothed and empiric values of C_{max} and t_{max} for each animal and PYR dose. The regression for animal 6WG on 7/8/93 predicted that the smoothed t_{max} would occur at 421 min, which was far outside the time range in which AChE-I was measured. This regression was rerun after omitting two unusually low AChE-I values observed at 90 and 105 min.

Table 5 presents descriptive statistics for smoothed and empiric C_{max} and t_{max} for all experiments of Phase IV conducted at 50 $\mu\text{g}/\text{kg}$ PYR. The sample size of 8 reflects the fact that four different animals were each tested on two days at this dose. The parameters for animal 6WG on 7/8/93 were based on the statistical results in which the two outlying data points were omitted.

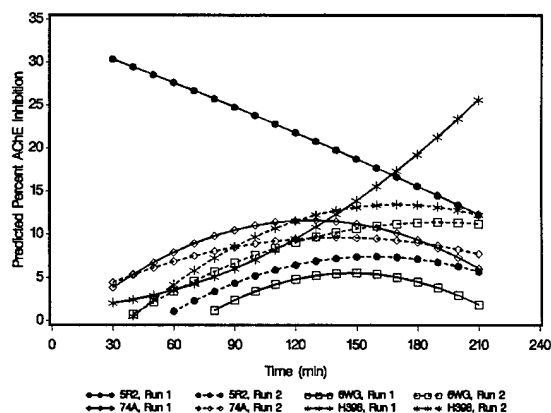
TABLE 5. DESCRIPTIVE STATISTICS FOR EMPIRICALLY OBSERVED AND QUADRATICALLY SMOOTHED VALUES OF C_{max} AND t_{max} FOR EXPERIMENTS PERFORMED WITH AN I.G. PYR DOSE OF 50 µg/kg^(a)

Variable	N	Minimum	Maximum	Mean	Std Dev
Empiric C _{max}	8	5.9	32.0	15.8	7.6
Smoothed C _{max}	8	5.5	30.3	14.0	8.3
Empiric t _{max}	8	30.0	180.0	125.6	50.0
Smoothed t _{max}	8	30.0	195.0	145.1	51.9

^(a)Using regression results for Animal 6WG on 7/08/93 which omitted 90 and 105 min values.

Figure 5 visually presents the smoothed quadratic curves for the four animals tested twice at 50 µg/kg PYR. These curves were generated using the fitted quadratic regression models. AChE-I values which were predicted to be negative are not shown. Two curves (animal 5R2 on 6/29/93 and animal H398 on 7/16/93) were atypical and were not downwardly concave within the time range evaluated. All of the other curves showed a smoothed t_{max} occurring between 120 and 210 min, with a smoothed C_{max} in the range of 5.5-13.5 percent.

FIGURE 5. PREDICTED PERCENT AChE INHIBITION VERSUS TIME FOR EACH EXPERIMENT AT 50 mg/kg



3.1.4.2 Lethality Studies

The second part of Phase IV was designed to determine if an i.g. PYR dose which resulted in RBC AChE-I similar to that produced by an effective i.m. dose of PYR provided equivalent protection from a 5 X 48-hr GD LD₅₀ challenge. It was estimated from results of the first part of this phase that an i.g. dose of approximately 40 µg/kg would result in 5-10 percent AChE-I at 150 min following PYR dosing. Therefore, two monkeys per study day had two baseline blood samples drawn approximately 5 min apart starting about 10 min prior to being given an i.g. dose of 40 µg/kg PYR. At approximately 135 and 145 min after PYR dosing, additional blood samples were obtained to measure AChE-I, and at 150 min following PYR administration, monkeys were challenged with 5 X GD LD₅₀.

Eight of the 10 monkeys given 40 µg/kg PYR i.g, injected 150 min later with 5 x GD LD₅₀, and treated with ATR/2-PAM survived for 48 hr. Forty-eight hour results for this group of animals are presented in Table D-9 in Appendix D.

Contingency table analyses were performed to confirm that the i.g. PYR dose did not significantly alter the survival rate from that of monkeys given i.m. PYR doses. Fisher's Exact Tests were used to statistically compare the survival rate of i.g. PYR-dosed animals with:

- 1) animals in the control group (0 PYR) of Phase III, and
- 2) all animals given an i.m. PYR dose of 4.0, 8.4, or 24.0 µg/kg in Phase III.

The conclusions drawn from the results of the contingency table analyses are:

- (1) The probability of survival is statistically greater for animals treated i.g. with 40 µg/kg PYR than that observed for the control group (0 PYR) of Phase III.
- (2) Survival rates were statistically equivalent for the i.g. and i.m.-dosed PYR groups.

A one-way analysis of variance was conducted to compare mean AChE-I levels at approximately 5 min prior to GD injection in monkeys from Phase III and Phase IV. Five groups of treated animals, the four i.m. PYR-dosed groups from Phase III and the i.g.

PYR group from Phase IV, were examined. Pairwise comparisons between groups of treated animals were performed using Tukey's Multiple Comparisons' procedure. Table 6 presents the results of these analyses. The mean AChE-I of 3.5 percent in the i.g. PYR group was statistically similar to the means of the 0 PYR control group (3.0 percent) and the i.m. 4.0 $\mu\text{g/kg}$ group (6.9 percent) of Phase III. The mean AChE-I in the i.g. 40 $\mu\text{g/kg}$ PYR monkeys was significantly lower than the means from the i.m. 8.4 $\mu\text{g/kg}$ PYR group (12.1 percent) and the i.m. 24 $\mu\text{g/kg}$ PYR group (28.7 percent).

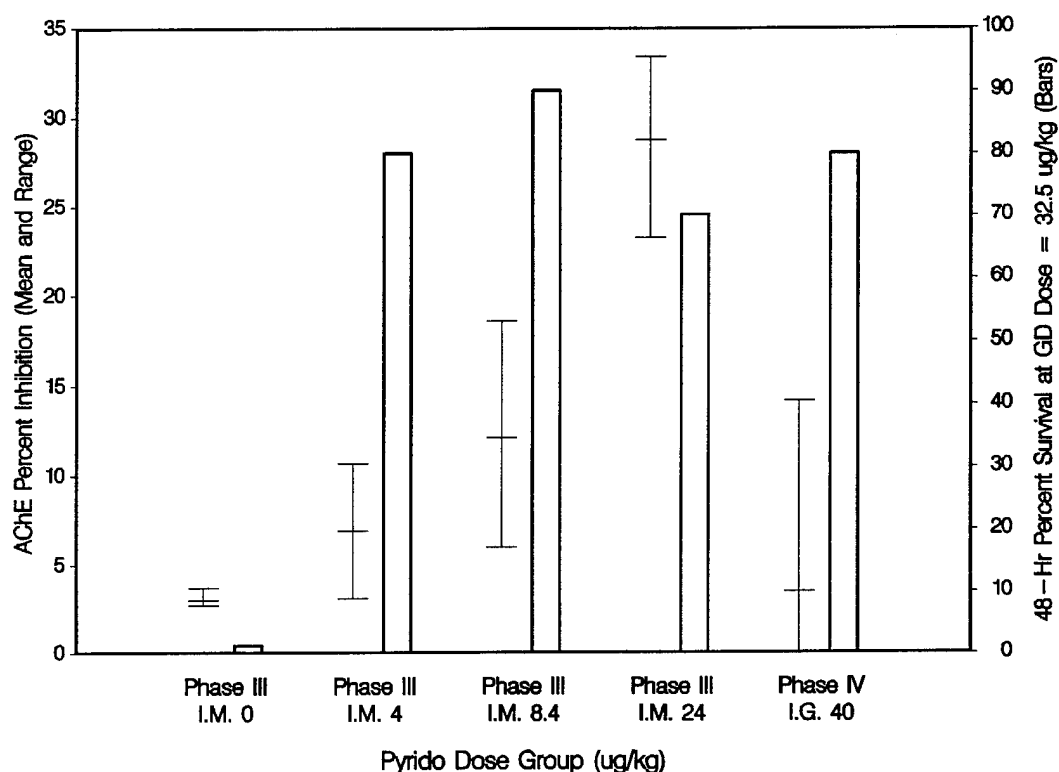
TABLE 6. ANALYSIS OF VARIANCE COMPARISON OF AChE-I FOR PHASE III (I.M.) AND PHASE IV (I.G.) EXPERIMENTS

Phase	PYR Dose ($\mu\text{g/kg}$), Route	N	Mean	(S.D.)	Tukey Grouping ^(a)
III	24 i.m.	10	28.7	(3.3)	A
III	8.4 i.m.	10	12.1	(3.5)	B
III	4 i.m.	10	6.9	(2.3)	B C
IV	40 i.g.	10	3.5	(6.3)	C
III	0 i.m.	4	3.0	(0.5)	C

^(a)Tukey's Studentized Range (HSD) Test for AChE-I conducted at the 95 percent level. Means with the same letter are not significantly different.

Figure 6 presents both AChE-I levels and survival rates for the five groups from the two experimental phases. PYR dose groups are displayed across the horizontal axis. Above each treatment group, the mean AChE-I (left vertical axis) for that group is shown bracketed by the low and high AChE-I levels. The proportion surviving (right vertical axis) is shown by the vertical bar. The plot clearly shows that the i.g. PYR group from Phase IV is similar to all PYR groups (4-24 $\mu\text{g/kg}$) from Phase III with respect to survival, but that it is most similar to the lowest two PYR groups (control and 4.0 $\mu\text{g/kg}$) with respect to AChE-I.

FIGURE 6. COMPARISON OF PERCENT AChE-I AT 5 MIN PRIOR TO GD INJECTION AND SURVIVAL RATES IN PHASES III AND IV



To summarize results of Phase IV studies, both pharmacodynamic and GD-induced lethality studies were conducted in animals treated i.g. with PYR. The results of the statistical analyses are as follows.

- (1) Eight of 10 monkeys given 40 $\mu\text{g/kg}$ PYR i.g., injected 150 min later with 5 x 48-hr GD LD_{50} , and treated with ATR/2-PAM survived for 48 hr.
- (2) The probability of survival was statistically greater for animals treated i.g. with 40 $\mu\text{g/kg}$ PYR than for the control group (0 PYR) of Phase III.
- (3) Although mean percent AChE-I in animals treated i.g. with 40 $\mu\text{g/kg}$ most closely resembled those levels observed in control animals and animals treated at the lowest i.m. PYR dose of 4 $\mu\text{g/kg}$, survival rates were statistically equivalent for the i.g. and all i.m. PYR dose groups.

3.1.5 Phase V

3.1.5.1 Lethality Results

Phase V studies began on August 31 and ended on October 26, 1993. The purpose of this phase was to evaluate the effect of adding 0.1 mg/kg diazepam (DZM) to the treatment regimen. Initially, GD dose-lethality response studies were planned for three treatment groups:

- PYR/ATR/2-PAM,
- PYR/ATR/2-PAM/DZM, and
- ATR/2-PAM/DZM.

The GD dose-lethality response curves for untreated animals and animals treated with ATR/2-PAM were estimated in Phase I. In Phase I, the 48-hr GD LD₅₀ for animals treated with ATR/2-PAM was estimated to be 20.5 µg/kg with 95 percent confidence limits of 16.2 and 26.2 µg/kg. Initial results of Phase V, after five animals were dosed in each treatment group, indicated that the 48-hr GD LD₅₀ for animals treated with ATR/2-PAM/DZM was substantially less than 20.5 µg/kg. To examine this apparent difference in efficacy between animals treated with ATR/2-PAM and animals treated with ATR/2-PAM/DZM, the protocol was amended to include an ATR/2-PAM treatment group in Phase V.

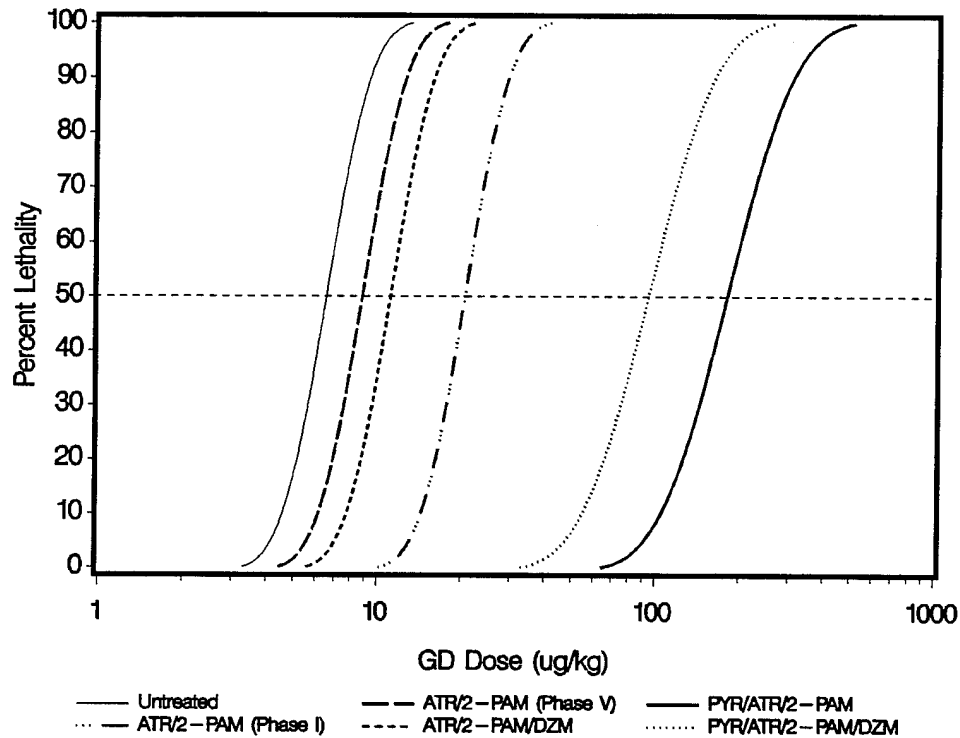
The total number of animals dosed in each treatment group of Phase V was 10 for each of the PYR/ATR/2-PAM, PYR/ATR/2-PAM/DZM, and ATR/2-PAM/DZM treatment groups, and eight for the ATR/2-PAM treatment group. Table D-10 in Appendix D presents results from all four of the Phase V treatment groups.

Probit dose-response models were fitted to the data to estimate the GD dose-lethality relations for each group of animals. Because historical results have shown that pretreatment with PYR flattens the slope of the GD dose-lethality curves, separate analyses were conducted for animals pretreated with PYR and animals not given PYR. First, a probit dose-

response model in log GD dose was fitted to the 48-hr lethality data for six groups of animals not treated with PYR: ATR/2-PAM, ATR/2-PAM/DZM, and the same four groups of animals modelled in Phase I as shown in Table 2. This model assumed that the GD dose-lethality curves for the six groups of animals had a common slope but different intercepts. The historical data from previously conducted MREF tasks were included to increase the precision of the estimated slope, thereby increasing the precision of the 48-hr GD LD₅₀ for each group of animals. The slope was estimated to be 8.7 with 95 percent confidence limits of 5.2 and 12.1. Second, a probit dose-response model in log GD dose was fitted to the 48-hr lethality data for two groups of PYR pretreated animals: PYR/ATR/2-PAM and PYR/ATR/2-PAM/DZM. This model also assumed that the GD dose-lethality curves for the two groups of animals had a common slope but different intercepts. The slope for the second model was estimated to be 5.7 with 95 percent confidence limits of 0.4 and 11.1.

Figure 7 displays predicted GD dose-lethality curves resulting from the two probit models. The six groups shown in Figure 7 are the untreated group (combined data from MREF Tasks 89-08, 89-12, and 92-30), the ATR/2-PAM group from Phase I, and the four groups of animals in Phase V. Table 7 displays the estimated 48-hr GD LD₅₀s, PRs, and corresponding 95 percent confidence intervals for each group of animals. The results demonstrate that the PR is significantly greater than one, at the 5 percent significance level, for each group of treated animals except for the eight animals treated with ATR/2-PAM in Phase V. The PR for this group of animals was estimated to be 1.4 with a 95 percent confidence interval of 1.0 to 1.8. LD₅₀ ratios, and confidence intervals, for each pair of Phase V treated animals are presented in Table 8. In addition, the LD₅₀ ratios for animals treated with ATR/2-PAM in Phase I and Phase V are also provided.

FIGURE 7. PREDICTED LETHALITY VERSUS GD DOSE FOR SIX GROUPS

TABLE 7. SUMMARY OF RESULTS OF PROBIT MODELS:
LD₅₀ VALUES AND PROTECTIVE RATIOS

Treatment Group	N	LD ₅₀	LD ₅₀ 95% Confidence Limits	Protective Ratio	Protective Ratio 95% Confidence Limits
Untreated ^(a)	25	6.5	(5.6,7.8)	1.0	(-)
ATR/2-PAM (Phase I)	10	20.6	(16.0,26.9)	3.2	(2.4,4.2) ^(b)
ATR/2-PAM/DZM	10	11.1	(7.9,15.3)	1.7	(1.2,2.4) ^(b)
ATR/2-PAM (Phase V)	8	8.8	(6.6,11.9)	1.4	(1.0,1.8)
PYR/ATR/2-PAM	10	182	(50.5,385)	27.8	(19.2,40.4) ^(b)
PYR/ATR/2-PAM/DZM	10	94.5	(29.1,252)	14.5	(9.7,21.7) ^(b)

^(a)Data for untreated groups from Tasks 89-08, 89-12, and 92-30 are combined.

^(b)Protective ratio is significantly greater than one ($p \leq 0.05$) since the lower confidence limit is greater than one.

TABLE 8. LD₅₀ RATIO COMPARISONS

Group One ^(a) (Denominator)	Group Two ^(a) (Numerator)	LD ₅₀		LD ₅₀ Ratio (95% C.L.)
		Group One	Group Two	Group Two/Group One
ATR/2-PAM	ATR/2-PAM (Phase I)	8.8	20.6	2.3 (1.6,3.4) ^(b)
ATR/2-PAM/DZM	ATR/2-PAM (Phase I)	11.1	20.6	1.9 (1.3,2.7) ^(b)
ATR/2-PAM	ATR/2-PAM/DZM	8.8	11.1	1.3 (0.8,1.9)
ATR/2-PAM	PYR/ATR/2-PAM	8.8	182	20.6 (13.3,31.9) ^(b)
ATR/2-PAM	PYR/ATR/2-PAM/DZM	8.8	94.5	10.7 (6.8,17.0) ^(b)
ATR/2-PAM/DZM	PYR/ATR/2-PAM	11.1	18.2	16.3 (10.4, 25.8) ^(b)
ATR/2-PAM/DZM	PYR/ATR/2-PAM/DZM	11.1	94.5	8.5 (5.3,13.7) ^(b)
PYR/ATR/2-PAM	PYR/ATR/2-PAM/DZM	182	94.5	0.5 (0.3,0.9) ^(c)

^(a)Treatment groups are from Phase V unless otherwise noted.

^(b)LD₅₀ ratio was determined to be statistically greater than one.

^(c)LD₅₀ ratio was determined to be statistically less than one.

Conclusions drawn from the LD₅₀ ratio comparisons, at the 5 percent significance level, are:

- 1) The 48-hr GD LD₅₀ for Phase V animals treated with ATR/2-PAM and ATR/2-PAM/DZM are not statistically different.
- 2) The 48-hr GD LD₅₀ for Phase I animals treated with ATR/2-PAM is statistically greater than those estimated for animals treated with ATR/2-PAM or ATR/2-PAM/DZM in Phase V.
- 3) The 48-hr GD LD₅₀s for Phase V PYR-pretreated animals are statistically greater than those estimated for Phase V animals not pretreated with PYR.
- 4) The estimated PRs for PYR-pretreated animals were greater than 14.
- 5) The 48-hr GD LD₅₀ for animals treated with PYR/ATR/2-PAM is statistically greater than that estimated for animals treated with PYR/ATR/2-PAM/DZM.

3.1.5.2 Effects of Cage Restraint

In Phase I, to minimize handling of the larger and more aggressive animals, monkeys were dosed while restrained in their cages, and ATR/2-PAM were injected in the same leg as the GD. In Phase V, however, animals were removed from their cages, placed on slotted V-shaped platforms with limbs restrained, transported to a hood, and injected with GD and

treatment prior to being returned to their cages. Because these different procedures may contribute to the difference in 48-hr GD LD₅₀s estimated for animals treated with ATR/2-PAM in Phases I and V, the protocol was amended and five additional animals were injected. These monkeys were injected, while restrained in their cages, at a fixed GD dose of 20.5 µg/kg, the 48-hr GD LD₅₀ estimated for ATR/2-PAM animals in Phase I, and injected with ATR/2-PAM in the same manner as in Phase I. Table D-11 in Appendix D displays the results for these animals. One of the five monkeys survived more than 48 hours.

Lethality results from the five animals of Phase V injected with GD and treated with ATR/2-PAM while restrained in cages were combined with the results from the other 8 animals treated with ATR/2-PAM in Phase V. A probit dose-response model was fitted to the lethality data for the Phase V ATR/2-PAM treated animals and five other groups of animals (ATR/2-PAM/DZM and the same four groups modelled in Phase I as displayed in Table 2). The data from the other five groups of animals were included to increase the precision of the estimated slope, thereby increasing the precision of the 48-hr GD LD₅₀ for the ATR/2-PAM treated animals. A dummy variable was included in the model to assess the statistical significance of cage restraint on lethality. The variable was set equal to one for the five animals in Phase V that were injected with GD and ATR/2-PAM while restrained in the cage and zero otherwise. The statistical significance of the estimated dummy variable permits an assessment of the impact of cage restraint on the lethality results.

The dummy variable was estimated to be -2.3 with a standard error of 1.1 and a 95 percent confidence interval of -4.4 to -0.3. Because the 95 percent confidence interval does not contain zero, the estimated dummy variable was determined to be significantly different from zero. Therefore, the lethality results of the five animals dosed while restrained in the cage are statistically incompatible with the GD dose-lethality relation estimated for the eight animals in Phase V that were injected with GD and ATR/2-PAM while on restraint platforms. While this result suggests that the different procedures contribute to the difference in 48-hr GD LD₅₀s estimated for ATR/2-PAM treated animals dosed in Phases I and V of this experiment, the significance is based on the survival of just one of the five animals, and therefore must be interpreted with caution. Additional experiments are recommended prior to reaching a definite conclusion on the effects of procedures on GD-induced lethality.

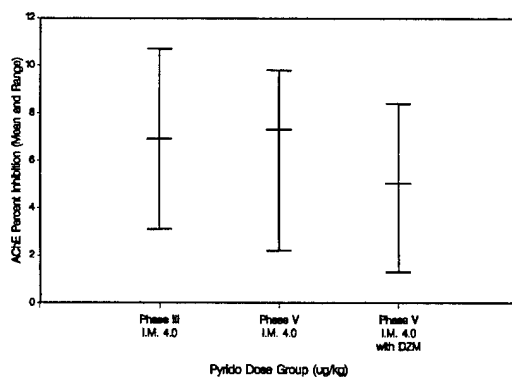
3.1.5.3 AChE-I Levels for Phase III and Phase V PYR Groups

Three groups of animals were treated with an i.m. PYR dose of 4.0 $\mu\text{g/kg}$ in MREF Task 92-30. Statistical comparisons were made between the AChE-I levels observed at 5 min prior to GD injection for these three groups of animals. Figure 8 presents AChE-I levels for each group. PYR dose groups are displayed across the horizontal axis, and above each group the mean AChE-I is shown bracketed by the minimum and maximum AChE-I levels measured. Means and standard deviations of AChE-I are shown below.

Phase	Treatment Group	N	AChE-I (%)	
			Mean	S.D
III	PYR/ATR/2-PAM	10	6.9	2.3
V	PYR/ATR/2-PAM	10	7.3	2.6
V	PYR/ATR/2-PAM/DZM	10	5.0	2.2

A one-way analyses of variance showed that the mean AChE-I values among the three groups were not different at the 5 percent significance level.

FIGURE 8. COMPARISON OF AChE-I LEVELS AT 5 MIN PRIOR TO GD INJECTION IN PHASES III AND V



3.1.5.4 Overall Lethality Results

GD dose-lethality experiments were conducted to estimate 48-hr survival. The results of the statistical analyses are:

- 1) There were no statistical differences between the 48-hr GD LD₅₀s estimated for animals treated in Phase V with ATR/2-PAM and ATR/2-PAM/DZM.
- 2) The 48-hr GD LD₅₀s for animals treated with ATR/2-PAM and ATR/2-PAM/DZM in Phase V were statistically less than that estimated for animals treated with ATR/2-PAM in Phase I.
- 3) The apparent difference between 48-hr GD LD₅₀s in ATR/2-PAM treated animals in Phases I and V may be due partially to the use of different experimental procedures for restraining and treating animals.
- 4) Although the addition of diazepam to the treatment regimen may have reduced the efficacy of PYR/ATR/2-PAM in preventing lethality (PR of 27.8), the PR for PYR/ATR/2-PAM/DZM was estimated to be 14.5 with 95 percent confidence limits of 9.7 and 21.7.

3.1.6 Statistical Analyses of Clinical Signs Data

A form was prepared for recording the incidence of specific clinical signs of intoxication or treatment following the injection of GD. The form had blocks for recording the occurrence, within specific time spans, of tremors, convulsions, excessive salivation/bronchial discharge, miosis, mydriasis, prostration or death. There were also blocks for recording whether an animal appeared normal or for supplying additional comments. In general, comments were very limited and were related primarily to when an animal was able to sit or stand following a period of prostration, or the occurrence of anisocoria, a condition in which the pupils are of unequal diameter. In one case, emesis was observed, and sometimes blood- or bile-tinged fluid was present under a cage. In one animal, blood was observed in the urine for several days, and one animal had a bloody discharge from the rectum. Another animal had tearing and a crusty lesion around an eye, presumably as a result of trauma to the area.

Clinical signs data of animals injected with GD were entered into a Paradox® database and statistically analyzed. The objectives of the statistical analysis of the clinical signs data were to:

- 1) Determine, for each clinical sign, whether a statistical relationship with GD dose existed.
- 2) Statistically compare the data for untreated and treated animals.
- 3) Statistically compare the data among the treated groups of animals.

3.1.6.1 Phase I

A listing of clinical signs data for Phase I animals is presented in Table D-12 of Appendix D. Table D-13 in Appendix D displays simple descriptive statistics for the Phase I ATR/2-PAM and untreated groups, using uncensored, nonmissing values. Means and standard deviations were calculated over the pooled data in each group without regard to GD dose.

Results of the statistical analysis for the Phase I clinical signs data are summarized in Table D-14. The proportion of animals displaying the sign in each group is shown in the second and third columns of the table and the p-value from the Fisher's Exact Test comparing these proportions is given in the fourth column. Column six contains the p-value from the Wilcoxon Test for comparing the mean durations for the two groups of animals. This analysis was conducted on the uncensored, nonmissing data for each group of animals, and did not consider the GD dose administered to each animal. The Wilcoxon Test was not performed on times to onset since they had been demonstrated to be GD dose-dependent.

Times to onset and durations within the first 2 hr and within the first 6 hr were analyzed by oneway ANOVAs with a covariate for log GD dose. The log GD dose slopes were generally significant for times to onset, but not for the durations within 2 hr and 6 hr. Therefore, durations were re-analyzed using oneway ANOVAs without a covariate for log GD dose. P-values for the log GD dose slopes are shown in the last column of Table D-14

for times to onset. Mean times to onset and durations, based on the ANOVAs, were predicted for each clinical sign. Because ANOVA models for times to onset included a covariate for log GD dose, mean predicted times to onset were computed at the 48-hr GD LD₅₀ for each group: 20.5 and 6.5 $\mu\text{g/kg}$ for ATR/2-PAM treated and untreated animals, respectively. A chi-square test was conducted to compare the mean predicted endpoints between ATR/2-PAM treated and untreated animals for each clinical sign. The chi-square p-values are displayed in column nine of the table.

Conclusions derived from the analysis of the Phase I clinical signs data, at the 5 percent significance level, are:

- 1) There are no statistical differences between incidence of clinical signs for ATR/2-PAM treated and untreated animals.
- 2) Log GD dose was significantly related to times to onset of tremors, convulsions, salivation, miosis, and prostration, and time to death, with times to onset predicted to decrease with increasing GD dose.
- 3) Mean predicted times to onset of convulsions, salivation, and miosis for ATR/2-PAM treated animals were significantly greater than those for untreated animals.
- 4) Mean predicted time to death for ATR/2-PAM treated animals was significantly greater than that for untreated animals.
- 5) Mean predicted duration of convulsions for ATR/2-PAM treated animals was significantly less than that for untreated animals.
- 6) Mean predicted duration of mydriasis within the first 2 hr for ATR/2-PAM treated animals was significantly greater than that for untreated animals.

3.1.6.2 Phase III

A listing of clinical signs data for Phase III animals is presented in Table D-15 in Appendix D. Table D-16 in Appendix D displays simple descriptive statistics for the PYR pretreated and untreated animals, using uncensored, nonmissing values.

There were no apparent differences among the clinical signs data for the three PYR dose groups. Data from the three PYR dose groups were pooled and all statistical comparisons were performed between two groups of animals: untreated and PYR pretreated animals. Table D-17 summarizes the results of the statistical analyses; information is analogous to that provided in Table D-14. Because Phase III animals were dosed at a fixed GD dose (5 x 48-hr GD LD₅₀), the ANOVA did not include a covariate for log GD dose.

Conclusions derived from the analysis of the Phase III clinical signs data, at the 5 percent significance level, are:

- 1) There are no statistical differences in incidence of clinical signs between PYR pretreated animals and untreated animals.
- 2) As discussed in Section 3.1.3, incidence of lethality for untreated animals was significantly greater than that for PYR pretreated animals. In addition, based on the ANOVA, mean predicted time to death for PYR pretreated animals was greater than that for untreated animals.
- 3) Mean predicted durations of tremors for PYR pretreated animals were significantly greater than those for untreated animals.
- 4) Mean predicted time to onset of miosis for PYR pretreated animals was significantly greater than that for untreated animals.
- 5) Durations of mydriasis for the untreated animals were significantly less, based on the Wilcoxon rank sum test, than those for PYR pretreated animals.

3.1.6.3 Phase IV

A data listing of clinical signs data for Phase IV animals is presented in Table D-18 in Appendix D. Animals in Phase IV were pretreated with an i.g. PYR dose of 40 µg/kg and challenged with the same GD dose as in Phase III, 5 x 48-hr GD LD₅₀. Statistical comparisons were made between Phase IV i.g. PYR pretreated animals and those injected with GD in Phase III. The groups of animals were numbered: (1) Phase III untreated animals, (2) Phase III i.m PYR pretreated animals, and (3) Phase IV i.g. PYR pretreated

animals. Table D-19 in Appendix D displays simple descriptive statistics for the three groups of animals.

Pairwise statistical comparisons were made between Phase IV i.g. PYR pretreated animals and Phase III untreated animals (Groups 1 and 3), and between Phase IV i.g. PYR pretreated animals and Phase III i.m. PYR pretreated animals (Groups 2 and 3). Group 1 and 2 comparisons were described in Section 3.1.6.2. Table D-20 summarizes the results of the statistical analyses and are comparable to those in Tables D-14 and D-17. Because Phase III and Phase IV animals were dosed at a fixed GD dose ($5 \times 48\text{-hr GD LD}_{50}$), the ANOVA did not include a covariate for log GD dose.

Conclusions derived from the comparisons of the Phase III and Phase IV clinical signs data, at the 5 percent significance level, are:

- 1) There are no statistical differences between incidence or duration of clinical signs for Phase IV i.g. PYR pretreated animals and Phase III i.m. PYR pretreated animals.
- 2) There are no statistical differences between incidence of clinical signs for Phase IV i.g. PYR pretreated animals and Phase III untreated animals.
- 3) As discussed in Section 3.1.4.2, lethality for Phase III untreated animals was significantly greater than that for Phase IV i.g. PYR pretreated animals. Mean predicted time to death for Phase IV i.g. PYR pretreated animals was greater than that for Phase III untreated animals.
- 4) Mean predicted duration of tremors for Phase IV i.g. PYR pretreated animals was significantly greater than that for untreated animals.

3.1.6.4 Phase V

A listing of clinical signs data for Phase V animals is presented in Table D-21 of Appendix D. Table D-22 in Appendix D displays simple descriptive statistics for the ATR/2-PAM, ATR/2-PAM/DZM, PYR/ATR/2-PAM, and PYR/ATR/2-PAM/DZM treated animals using uncensored, nonmissing values. Means and standard deviations were calculated over the pooled data in each group without regard to GD dose.

Table D-23 summarizes the results of the statistical analysis for the Phase V clinical signs data, and are comparable to those in Tables D-14, D-17, and D-20. Clinical signs information from the 5 animals injected with GD while restrained within their cages and treated with ATR/2-PAM were not included in the statistical analysis. Comparisons of the incidence of clinical signs between PYR pretreated monkeys and animals not pretreated were made using Fisher's Exact Test. Similarly, durations of clinical signs were compared between these two groups of animals using Wilcoxon's Test. Times to onset and durations were analyzed by oneway ANOVAs; a covariate for log GD dose was included in the ANOVAs for times to onset. Because ANOVA models for times to onset included a covariate for log GD dose, mean predicted times to onset were computed at the 48-hr GD LD₅₀ for each group: 8.8 µg/kg, 11.1 µg/kg, 182 µg/kg, and 94.5 µg/kg for ATR/2-PAM, ATR/2-PAM/DZM, PYR/ATR/2-PAM, and PYR/ATR/2-PAM/DZM treated animals, respectively.

Conclusions derived from the analyses of the Phase V clinical signs data, at the 5 percent significance level, are:

- 1) Mean predicted incidence and duration of salivation, miosis, and prostration for PYR pretreated animals were statistically greater than those for animals not given a PYR pretreatment.
- 2) Mean predicted duration of mydriasis for PYR pretreated animals was statistically less than that for animals not given a PYR pretreatment.
- 3) Log GD dose was significantly related to times to onset of convulsions, salivation, miosis, and prostration, and time to death, with times to onset predicted to decrease with increasing GD dose.
- 4) Mean predicted time to death for PYR pretreated animals was significantly greater than that for animals not given a PYR pretreatment.

3.2 Pathology

Few macroscopic changes were observed during necropsy of the 103 monkeys. Hemorrhage or potential hemorrhage (red or dark fluid or discoloration), which may be related to agent-induced toxicity, was seen in the lungs, urinary bladder and/or heart of eight of the monkeys. Hemorrhage, or potential hemorrhage, was also seen in the intestine or stomach of several monkeys. Gastrointestinal hemorrhage may be associated with agent toxicity, or may be associated with pyridostigmine pretreatment.

Occasional monkeys exhibited minor contusions, usually on the head, indicative of minor trauma due to tremors or convulsions. Massive, conspicuous morphologic alterations were not present in any of the monkeys, and death for all spontaneous-death animals was attributed to pharmacologic actions of soman.

Several animals had minor tracts of necrosis along the sites of test- or therapy-agent injection. These tended to occur only in animals which survived several days before necropsy. Other lesions seen grossly were typical of incidental parasitic diseases or anatomic variations which were unrelated to the test or therapy agents or test procedures. Gross pathology observed at the time of necropsy of each animal is presented in Appendix C.

Animals sacrificed were perfused with neutral buffered formalin and tissue samples placed in formalin. Tissues from animals that died on study were fixed in formalin. Tissues harvested were sent to USAMRICD for histopathologic evaluation.

4.0 CONCLUSIONS

The primary objective of this task was to determine the minimum dose of pyridostigmine bromide effective in protecting rhesus monkeys from lethality following a 5 X GD LD₅₀ challenge. Secondary objectives were to determine:

- 1) the relationship between PYR dose and RBC AChE-I;
- 2) the relationship between RBC AChE-I induced by PYR and lethality in monkeys challenged with 5 X GD LD₅₀ and treated with ATR/2-PAM;

- 3) the effect on severity of GD intoxication of adding 0.1 mg/kg diazepam to the ATR/2-PAM therapy; and
- 4) treatment efficacy of an intragastrically-administered dose of PYR which creates an RBC AChE-I level comparable to those observed following an effective i.m. dose of PYR.

Phase I experiments were conducted to determine the 48-hr GD LD₅₀ value for untreated monkeys and the protective ratio provided monkeys treated with ATR/2-PAM. The slope of the GD dose-lethality curve was estimated to be 12.1 and the 48-hr GD LD₅₀ 6.5 µg/kg. The estimated LD₅₀ for ATR/2-PAM treated monkeys of Phase I was 20.5 µg/kg, and the PR of 3.2 was significantly higher than that previously estimated in Task 85-18.

Experiments were conducted in Phase II to estimate the i.m. PYR dose which would produce a mean peak 23 percent AChE-I, and the time following PYR dosing at which the peak occurs. The PYR dose producing a mean peak AChE-I of 23 percent was estimated to be 24.2 µg/kg. A PYR dose of 8.4 µg/kg was estimated to produce a smoothed mean peak AChE-I of 9.4 percent. There were no recognizable PYR dose-related differences in empiric or smoothed t_{max}. The mean empiric t_{max}, averaged over all i.m. PYR dose groups, was 42.3 min.

Dose-response experiments with varying doses of i.m. PYR were conducted in Phase III to determine the PYR dose-lethality relationship for monkeys injected with 5 X 48-hr GD LD₅₀ at the time of predicted maximum PYR-induced AChE-I. Thirty-four animals were tested at one of four PYR doses (0, 4.0, 8.4, or 24.0 µg/kg). Survival rates were significantly greater for each of the PYR-dosed groups relative to the control group, and survival rates were not statistically different among the three PYR-dosed groups. A one-way analyses of variance demonstrated that average AChE-I levels among the PYR dose groups were significantly ($p < 0.0001$) different, with an increasing dose-response relationship.

Pharmacodynamic studies were performed to measure the effects of intragastrically-administered PYR on AChE-I levels in Phase IV. It was estimated that an i.g. dose of

approximately 40 $\mu\text{g/kg}$ would result in 5-10 percent AChE-I at 150 min following PYR dosing. Monkeys were given 40 $\mu\text{g/kg}$ PYR intragastrically and challenged with 5 X 48-hr GD LD₅₀. Survival rates were statistically equivalent for the i.g. and i.m.-dosed PYR groups.

Experiments were conducted in Phase V to evaluate the effect of adding 0.1 mg/kg diazepam (DZM) to the treatment regimen. GD dose-lethality response studies were conducted for four treatment groups: PYR/ATR/2-PAM; PYR/ATR/2-PAM/DZM; ATR/2-PAM/DZM; and ATR/2-PAM. Conclusions drawn from the estimated probit dose-response models, at the 5 percent significance level, are:

- 1) The 48-hr GD LD₅₀ for Phase V animals treated with ATR/2-PAM or ATR/2-PAM/DZM are not statistically different.
- 2) The 48-hr GD LD₅₀s for Phase V PYR-pretreated animals are statistically greater than those estimated for Phase V animals not pretreated with PYR.
- 3) The estimated PRs for PYR-pretreated animals were greater than 14. Although the addition of diazepam to the treatment regimen may have reduced the efficacy of PYR/ATR/2-PAM in preventing lethality (PR of 27.8), the PR for PYR/ATR/2-PAM/DZM was estimated to be 14.5 with 95 percent confidence limits of 9.7 and 21.7.

In Phase I, heavier and more aggressive monkeys were dosed while restrained in their cages, and ATR and 2-PAM were injected in the same limb as the GD. In Phase V, however, animals were removed from their cages, placed on slotted V-shaped platforms with limbs restrained, transported to a chemical fume hood, and injected with GD and treatment prior to being returned to their cages. Because these different procedures may contribute to the apparent difference in 48-hr GD LD₅₀s estimated for animals treated with ATR/2-PAM in Phases I and V, five animals were injected, while restrained in their cages, at a fixed GD dose and treated with ATR/2-PAM. The lethality results using these five animals was statistically incompatible with the GD dose-lethality relation estimated for ATR/2-PAM treatment for animals restrained on platforms. This result suggests that the methods of restraint and treatment may contribute to the difference in 48-hr GD LD₅₀s estimated for ATR/2-PAM treated animals in Phases I and V of this experiment.

5.0 DISCUSSION

The administration of PYR prior to injection of GD greatly flattens the slope of the GD dose-lethality response curve, and prevents the determination of a minimum effective PYR dose. Small doses of PYR, given i.m. or i.g., and at times producing RBC AChE-I virtually indistinguishable from that of untreated controls, is effective in reducing GD-induced lethality. Intragastric administration of PYR at a dosage that creates RBC AChE-I equivalent to an effective intramuscular dose appears to be equally efficacious. Route of administration does not appear to affect efficacy of PYR as long as equivalent levels of RBC AChE-I are attained. There is a definite PYR dose-AChE-I response, with increasing AChE-I with increasing doses of PYR. PYR appears to be effective in reducing lethality from GD injection at dosage levels much less than previously tested.

The protocol of this study originally contained a phase to examine possible mechanisms of protection of PYR other than AChE-I. This was to be accomplished by waiting until PYR-induced decreased levels of AChE activity were back to a baseline level prior to challenge with GD. In preliminary work with guinea pigs at USAMRICD, however, this approach to testing demonstrated no protection to GD injection from PYR pretreatment once RBC AChE activity had returned to a normal level. Therefore, the Task 92-30 protocol was amended and a similar study using monkeys was not attempted.

There is no obvious explanation for the observed difference in 48 hr LD₅₀s of ATR/2-PAM treated animals between Phases I and V. In Phase I, to avoid handling the larger and more aggressive animals, monkeys were injected with GD and ATR/2-PAM, in the same limb, while restrained within a "squeeze-back" cage. In Phase V, animals were restrained on a tie-down board, injected with GD while within a chemical fume hood, and injected with ATR/2-PAM in the other hind limb. To assess the extent to which the difference in procedures affected lethality results, 5 monkeys were injected with 20.5 µg/kg GD, the predicted GD LD₅₀ for ATR/2-PAM treated animals in Phase I, in the gastrocnemius muscle while restrained within a cage and treated with ATR/2-PAM one minute later by injection into the quadriceps muscle of the same leg. The GD LD₅₀ of Phase V animals

challenged while on a restraint board and treated with ATR/2-PAM was 8.8 $\mu\text{g}/\text{kg}$. If 8.8 $\mu\text{g}/\text{kg}$ is the true GD LD_{50} for animals treated with ATR/2-PAM, then the probability of lethality for animals injected with a 20.5 $\mu\text{g}/\text{kg}$ GD dose is approximately 99.9 percent. Thus, the likelihood of one or more of the five animals surviving 48 hr at this GD dose is very remote, less than 1 percent. However, one of the five animals so treated did survive for more than 48 hr. Therefore, the true GD LD_{50} of ATR/2-PAM treated animals challenged while restrained within a cage is probably greater than that of ATR/2-PAM treated animals challenged while on a tie-down board. Injecting ATR/2-PAM into the same limb as GD could influence treatment efficacy. Also, monkeys are relatively familiar with the procedure of being "squeezed" within a cage to receive injections. By performing injections with GD and ATR/2-PAM in this manner, it is probable that stress levels were considerably less than those produced by the catching and restraining procedures used in Phase V. This could have an effect on neurotransmitter and cortisol⁽¹²⁾ release which could affect the efficacy of therapy. It is important to note, however, that the LD_{50} of the Phase I untreated group of monkeys injected with GD while in a restraint cage was comparable to i.m GD LD_{50} s estimated in previous studies at the MREF where tie-down boards were used.

A two or three fold difference in results between biological studies is not at all uncommon, but was unexpected in this experiment. The difference observed in this study does exemplify the risks taken when combining data from studies where seemingly minor modifications in technique were used.

Although the addition of diazepam to the ATR/2-PAM therapy in PYR-pretreated monkeys may decrease the PR from GD, the estimated PR from GD for PYR-pretreated, ATR/2-PAM/DZM treated monkeys was still approximately 15. The addition of diazepam to therapy following GD injection has been shown to decrease the incidence of convulsions and decrease the pathology observed in the central nervous system.^(13,14,15)

6.0 RECORD ARCHIVES

Records pertaining to the conduct of this study are contained in Battelle laboratory record books which are specific for this task. These records and the final report will be archived at Battelle. Agent dosing solutions are unstable under prolonged storage and have been destroyed. Samples of PYR, Mestison®, ATR, 2-PAM, and diazepam will be maintained at the MREF. Tissue samples collected at necropsy have been sent to USAMRICD.

7.0 ACKNOWLEDGEMENTS

The names, titles, and degrees or certification of the principal contributors to this study are listed below:

<u>Name</u>	<u>Title</u>	<u>Degree</u>
Dr. Carl T. Olson	Study Director	D.V.M., Ph.D.
Dr. Ronald G. Menton	Study Statistician	Ph.D.
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8.0 REFERENCES

- (1) Joiner, R.L., and Kluwe, W.M., Task 85-18: Conduct of Pralidoxime Chloride, Atropine in Citrate Buffer and Pyridostigmine Bromide Pharmacokinetics Studies, and Comparative Evaluation of the Efficacy of Pyridostigmine Plus Atropine and Pralidoxime Versus Atropine and Pralidoxime Alone Against Acute Soman Poisoning in Male Rhesus Monkeys, Final Report to U. S. Army Medical Research and Development Command, Institute of Chemical Defense, August 1988.
- (2) Contract No. DAMD17-83-C-3129. Letter dated 25 July 1988, from Battelle to Commander, U.S. Army Medical Research Acquisition Activity, Ft. Detrick, MD, regarding results of Task 87-34: The Effect of Treatment Regimens of Variable Concentrations of Atropine Sulfate in Combination with Pralidoxime Chloride on the Survival of Soman-Challenged Rhesus Monkeys Pretreated with Pyridostigmine Bromide.
- (3) Feder, P.I., Hobson, D.W., Olson, C.T., Joiner, R.L., and Matthews, M.C., Chapter 4 "Statistical Design Considerations for Stagewise, Adaptive Dose Allocation in Dose-response Studies", in Biopharmaceutical Sequential Statistical Applications, K.E. Peace, ed., Marcel Dekker Inc., New York, NY, 1992.
- (4) Method No. 17/In Vitro, Method for Determination of Monkey Erythrocyte Acetylcholinesterase (AChE) Activity in Laboratory Animal Blood with the COBAS FARA Centrifugal Analyzer, Medical Research and Evaluation Facility, Battelle, February 19, 1993.
- (5) Finney, D.J., Probit Analysis, Third Edition, Cambridge University Press, Cambridge, England, 1971.
- (6) Nelson, W.B., Applied Life Data Analysis, John Wiley and Sons, New York, NY, 1982.

- (7) Zar, J.H., Biostatistical Analysis, Prentice-Hall, Englewood Cliffs, NJ, 1984.
- (8) Lehmann, E.L., Nonparametrics: Statistical Methods Based on Ranks, Holden-Day, San Francisco, CA, 1975.
- (9) Lawless, J.E., Statistical Models and Methods for Lifetime Data, John Wiley and Sons, New York, NY, 1982.
- (10) Olson, C.T., Dill, G.S., Menton, R.G., Kiser, R.C., Hayes, T.L., Snider, T. H. and Singer, A.W. Task 89-08: An Efficacy and Pharmacokinetic Evaluation of a Dose of Diazepam that will Reduce the Incidence of Convulsions in Indian Rhesus Monkeys Pretreated with Pyridostigmine Bromide, Challenged with Soman, and Treated with Atropine and Pralidoxime Chloride with the Diazepam. Final Report to U.S. Army Medical Research and Development Command, Institute of Chemical Defense December 1990.
- (11) Olson, C.T., Menton, R.G., Kiser, R.C., Matthews, M.C., Hayes, T.L., Singer, A.W., and Dill, G.S. Task 89-12: Characterization of Soman Toxicity in Atropine and Oxime (HI-6 and MMB-4) Treated Rhesus Monkeys. Final Report to U.S. Army Medical Research and Development Command, Institute of Chemical Defense, March 1991.
- (12) Levine, B.S., Matamoros, R.A., Influence of Stress on the Toxicity and Metabolism of Organophosphorus Insecticides, *The Toxicologist*, Vol 15, March 1995.
- (13) Olson, C.T., Dill, G.S., Menton, R.G., Kiser, R.C., Hayes, T.L., Snider, T.H., and Singer, A.W., Task 89-08: An Efficacy and Pharmacokinetic Evaluation of a Dose of Diazepam that will Reduce the Incidence of Convulsions in Indian Rhesus Monkeys Pretreated with Pyridostigmine Bromide, Challenged with Soman, and Treated with Atropine and Pralidoxime Chloride with the Diazepam. Final Report to U.S. Army Medical Research and Development Command, Institute of Chemical Defense, December 1990.
- (14) Wall, H.G., Jaax, N.K., and Hayward, I.J., Brain Lesions in Rhesus Monkeys after Acute Soman Intoxication, Proceedings of the Sixth Medical Chemical Defense Biosciences Review, Commander, USAMRICD, Aberdeen Proving Ground, MD, 1987.
- (15) Proceedings of the Workshop on Convulsions and Related Brain Damage Induced by Organophosphorus Agents, sponsored by the U.S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD, 1-2 February 1990.

APPENDIX A

MREF PROTOCOL 88

Determination of the Minimum Effective Pyridostigmine Pretreatment Dose
in Monkeys Challenged with 5XLD₅₀ Soman and Treated with Atropine/2-PAM

Study Performed by Battelle Memorial Institute
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7. Sponsor: U.S. Army Medical Research and Development Command (USAMRDC)
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Research Institute of Chemical Defense (USAMRICD)
9. Introduction:

Current U.S. Army therapy for countering exposure to the organophosphonate pinacolylmethylphosphonofluoridate (soman; GD) is pretreatment (administration prior to exposure) with the carbamate pyridostigmine bromide (PYR) and treatment following exposure with atropine and pralidoxime chloride (2-PAM). The minimum effective dosage of PYR has not been established. In previous experiments conducted at the MREF, rhesus monkeys given 1.2 mg/kg PYR by nasogastric intubation every eight hr for a total of four doses prior to GD, and atropine and 2-PAM therapy following exposure to GD, were effectively protected. Pretreatment with PYR, in conjunction with atropine and 2-PAM treatment, was shown to provide significantly improved protection from GD-induced lethality than atropine and 2-PAM therapy alone. Additional research is necessary to determine the minimum effective PYR dose, the relationship of erythrocyte (RBC) acetylcholinesterase (AChE) inhibition (AChE-I) to PYR dose, and the effect of adding an anticonvulsant, such as diazepam, to the treatment regimen.

10. Objective:

The primary objective of the proposed research is to determine the minimum effective dose (MED) of PYR, and the associated RBC AChE-I level,

for protection from 5 X GD LD₅₀ (5 times the GD dose lethal to 50 percent of challenged, untreated monkeys). For this study, the MED of PYR is defined as the minimum dose of PYR which provides a 95 percent survival rate in monkeys injected with five times the GD LD₅₀ and treated with 0.4 mg/kg atropine and 25.7 mg/kg 2-PAM. This study will be conducted following the guidelines of the Food and Drug Administration (FDA) Good Laboratory Practice (GLP) Act. (SC920203)

Secondary objectives of the study are to determine:

- a. the relationship between RBC AChE-I and PYR dosage;
- b. the relationship between RBC AChE-I, induced by PYR, and lethality in monkeys exposed to 5 X the GD LD₅₀ and treated with atropine/2-PAM;
- c. the effect on the severity of GD intoxication of adding 0.1 mg/kg diazepam to the atropine/2-PAM therapy; and
- d. treatment efficacy of multiple oral doses of PYR creating AChE-I levels in monkeys comparable to those observed for the intramuscular (i.m.) MED of PYR.

11. Experimental Design:

A. Test System

- (1) Animals - Male rhesus monkeys, Macaca mulatta, of Indian origin were specified for this study because there is considerable scientific evidence that the monkey is predictive of GD therapeutic responses in man. Rhesus monkeys of Indian origin were selected because the majority of work in this area has been done with monkeys of Indian origin, and because there is evidence that rhesus monkeys of Chinese origin respond somewhat differently to these study conditions than those of Indian origin.⁽¹⁾ Monkeys for use in this study will be provided by USAMRICD. Experiments are conducted in a stage-wise fashion to limit the number of animals used to the minimum necessary to achieve statistically valid results. Monkeys are observed for 10 days following exposure. Discomfort and injury of animals are limited to that which is unavoidable in the conduct of scientifically valuable research. If, in the opinion of the Study Veterinarian or the Study Director, a monkey appears to be in a moribund state and in pain, that animal will be anesthetized with sodium pentobarbital or other approved anesthetic solution, perfused with formalin, and a complete necropsy performed with tissues taken for histologic evaluation. Anesthetics, analgesics, or tranquilizers cannot be used for the relief of pain or anxiety in these studies because they could

interfere with the biological effects of the challenge agent or therapy compounds. External stimuli and manipulation are minimized to decrease any associated anxiety. Protocols of all experiments using animals are reviewed and approved by Battelle's Institutional Animal Care and Use Committee (IACUC) prior to initiation of the study. The proper care and use of animals in the conduct of research described in this protocol is the responsibility of the Study Veterinarian, the Study Director, and MREF management.

- (2) Initial Weight - Monkeys placed on study weigh between approximately 4 and 7 kg.
- (3) Quarantine - All primates received at Battelle routinely undergo a 1.5 month quarantine period. All animals are examined by the Study Veterinarian within one week of arrival at Battelle. Blood samples are taken for hematology and serum chemistries. Fecal samples are taken for parasite infestation evaluation. Three tests for the presence of tuberculosis are performed by injecting tuberculin intradermally in the palpebral skin at 2 week or longer intervals.
- (4) Animal Selection - Based on physical examinations and clinical laboratory findings, acceptable animals are identified by the Study Director and Study Veterinarian. Because chemical restraint cannot be used during these studies, the larger, more aggressive animals will be used to estimate median lethal doses of GD, with and without specific treatments, by injecting these animals while restrained within cages in an animal holding area.
- (5) Animal Identification - Animals are received with tattoos. If a monkey arrives without a tattoo or with an identification number that duplicates another animal's, a new tattoo will be applied.
- (6) Housing - Monkeys are housed individually in stainless-steel cages, approximately 24 inches wide, 34 inches high, and 26 inches deep, with automatic watering systems.
- (7) Acclimation - Prior to the start of experiments, monkeys are acclimated to the type of restraint to be used. This includes restraint chairs and/or slotted, V-shaped platforms where arms and legs can be restrained by means of lanyards.
- (8) Lighting - Fluorescent lighting is used with a light/dark cycle of 12 hr each per day.

- (9) Temperature - Monkey room temperatures are maintained at 65 to 84 F. At least 90 percent of the total recordings will fall within the specified range.
- (10) Humidity - Relative humidity of monkey rooms is maintained at 30 to 70 percent. At least 90 percent of the total recordings will fall within the specified range.
- (11) Diet - Purina certified monkey chow biscuits are fed twice daily and may be periodically supplemented with fresh fruit or primate treats (including a certificate of analysis). No contaminants that would interfere with the results of the study are known to be present in the feed. Analyses of the feed can be obtained from Purina.
- (12) Water - Water is supplied from the Battelle water system and given ad libitum through automatic watering systems. No contaminants that would interfere with the results of the study are known to be present in the water. Water is analyzed for potability and for contaminants annually.
- (13) Battelle's Animal Resources Facilities have been registered with the U.S. Department of Agriculture (USDA) as a Research Facility (Number 31-R-021) since August 14, 1967, and are periodically inspected in accordance with the provisions of the Federal Animal Welfare Act. In addition, animals for use in research are obtained only from laboratory animal suppliers duly licensed by the USDA. Battelle's statement of assurance regarding the Department of Health and Human Services (DHHS) policy on humane care of laboratory animals was accepted by the Office of Protection from Research Risks, National Institutes of Health on August 27, 1973. Animals at Battelle's MREF are cared for in accordance with the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" (DHHS Publication No. (NIH) 85-23) and/or in the regulations and standards as promulgated by the Agricultural Research Service, USDA, pursuant to the Laboratory Animal Welfare Act of August 24, 1966 as amended (P.L. 89-544 and P.L. 91-579).
- (14) On January 31, 1978, Battelle received full accreditation of its animal care programs and facilities from the American Association for Accreditation of Laboratory Animal Care (AAALAC). Battelle's full accreditation status has been renewed after every inspection since the original accreditation. The MREF is a part of the facilities granted full accreditation.

B. Test Material

- (1) Test Compounds - Pretreatment and treatment compounds, pyridostigmine bromide, atropine, 2-PAM, and diazepam are provided by USAMRICD or procured from commercial sources. Identity and purity analyses of compounds provided by the U.S. Army will be furnished with each shipment and will not be duplicated by Battelle chemists. Analyses for identity, purity, and concentration of solutions of compounds acquired from commercial sources will be accomplished by Battelle. Analyses for concentration of commercial solutions will be accomplished prior to and following completion of the study to confirm stability.
- (2) Chemical Agent - GD is supplied by USAMRICD. Purity, appropriate identification (batch number, lot number, state), and stability data are provided by USAMRICD. Purity and stability of agent stored at Battelle is periodically confirmed by Battelle personnel. A dosing solution of GD will be prepared prior to study initiation, aliquotted in approximately 5 mL quantities into vials, and frozen at approximately -70 C. The GD dosing solution will be analyzed using gas chromatography to confirm desired concentration after preparation. On each day that an aliquot of the GD dosing solution is used to inject monkeys, a sample will be taken and chemical analysis accomplished. If necessary to biologically confirm the agent potency, the 30 min and 24 hr GD LD₅₀s can be determined in mice using 5 doses of GD with 6 mice per dose group, as described in MREF Protocol 78.
- (3) Surety, security, and safety procedures for the use of chemical agents are thoroughly outlined in facility plans, in personnel requirements for qualification to work with chemical surety materiel (CSM), and in standard operating procedures for storage and use of CSM.

C. Test Groups

Experiments will be performed in phases, using results of previous phases to assist in the selection of PYR doses for succeeding phases and to reduce the number of animals required to obtain scientifically meaningful data.

(1) Initial Tests to Confirm LD₅₀s

No more than 10 monkeys are used to approximate the 48 hr GD LD₅₀ in animals given no treatment. This is accomplished using a modified up-and-down experimental design, challenging 1 or

2 monkeys per day and increasing or decreasing the GD dosed based on results obtained to date, and assuming a GD dose-lethality response slope consistent with results from earlier Battelle experiments with monkeys. If, after 4 or more monkeys have been challenged, the estimated GD LD₅₀ in this study falls within the 95 percent confidence limits of the recent historic Battelle GD LD₅₀ in Indian rhesus monkeys, the historic LD₅₀ will be accepted for this group of animals. An estimate of the GD LD₅₀ in up to 10 Indian rhesus monkeys given atropine (0.4 mg/kg) and 2-PAM (25.7 mg/kg) therapy 1 min following injection of GD will also be determined. This will be accomplished in a similar up-and-down type of experiment. If the 48 hr protective ratio (PR; LD₅₀ of treated animals/LD₅₀ of untreated animals) falls within 1.4-1.9 after 4 or more monkeys have been challenged and treated, this phase of the study will end. Because chemical restraint cannot be used during these studies, the larger (> 6 kg), or more aggressive animals will be used to estimate median lethal doses of GD, with and without atropine/2-PAM treatment, by injecting these animals while restrained within cages in an animal holding area. At the end of all phases of this experiment, if unexposed animals are available, an estimate of the GD LD₅₀ following treatment with atropine/2-PAM and 0.1 mg/kg diazepam will also be obtained. This will likewise be accomplished in an up-and-down manner, exposing only a few monkeys at a time.

- (2) Estimation of the i.m. PYR dose required to produce a 23 percent mean peak RBC AChE-I

Two monkeys will be injected with 10.5 µg/kg of PYR and blood samples taken at -5, 5, 10, 20, 30, 45, 60, and 90 min after PYR injection. Blood is separated into cells and plasma by centrifugation and the packed RBCs analyzed for AChE activity using an automated centrifugal chemical analyzer. Depending upon the peak RBC AChE-I observed during this time period, PYR doses may be altered in subsequent studies to obtain an estimate of the PYR dose which causes a peak AChE-I level approximating 23 percent. Animals studied in this phase will be used again after a minimum one week washout period, and can also be used in later phases of the experiment. Ten monkeys will be used to determine the i.m. PYR dose resulting in a mean peak 23 percent RBC AChE-I.

Two of the same monkeys will be injected with a PYR dose 0.45 log units below the dose which produces a mean peak 23 percent AChE-I to determine whether or not AChE-I at this lower PYR dose is significantly greater than zero and if time to peak AChE-I differs from that with the higher dose. If

significant AChE-I is obtained, the remaining eight animals used in the original study will also be injected with the same PYR dosage and blood samples will then be collected. If significant AChE-I is not obtained with the lower dose, the PYR dose will be increased by 0.15 log units and the study repeated.

To perform this phase of the study, each monkey is acclimated to a restraint chair prior to study initiation. An indwelling catheter is placed in a saphenous vein, and the monkey restrained in a chair while injected with PYR and during the 90 min period in which blood samples are taken.

(3) Estimation of the i.m. PYR MED for a 5 X GD LD₅₀ Challenge

Stage-wise designed experiments are conducted, with varying doses of i.m. PYR, to determine the PYR dose-lethality response slope and PYR ED₅₀ (PYR dose effective in preventing lethality in 50 percent of animals challenged) and MED for monkeys injected with 5 times the GD LD₅₀ at the time of predicted maximum PYR-induced AChE-I, and treated with 0.4 mg/kg atropine and 25.7 mg/kg 2-PAM at 1 min following GD injection. Blood samples will be taken prior to PYR injection and just prior to GD injection to determine the RBC AChE-I level. Early stages of the experiment will focus on high and low doses of PYR to ensure that lethality is observed in monkeys given low doses of PYR and that monkeys given sufficiently high doses of PYR survive. Initially, using two monkeys at each PYR dose, monkeys will be treated with the PYR dose predicted to produce 23 percent AChE-I, and at three additional lower doses at 0.15 log unit intervals. In all subsequent stages, doses of PYR used will depend upon survival observed in previous stages. Monkeys are observed continuously for the first two hours following GD injection and at decreasing frequency thereafter for a total of 10 days.

At the desired time after PYR dosing, a monkey is removed from his cage, and placed on a slotted, V-shaped platform with arms and legs restrained. They are then transported to a hood approved for the use of highly hazardous materials. GD will be injected, with the monkey within the hood, in the right leg in the posterior tibial area in the region of the gastrocnemius muscle at a site clipped of hair and pre-marked. The site of GD injection will be decontaminated with a 5 percent hypochlorite solution followed by a water rinse and the monkey removed from the hood. Atropine and 2-PAM will be injected intramuscularly in succession at separate sites 2 to 3 cm distant from each other in the Quadriceps femoris muscle of the left leg. To obtain maximum accuracy in the measurement of delivered doses,

syringes used for dosing will be Hamilton microliter syringes of the smallest compatible volume. Syringes are filled to no more than 95 percent of labeled total volume. Individual, labeled syringes are loaded with the calculated volume of GD dosing solution prior to the start of dosing, weighed and placed on ice until used. After dosing is accomplished, syringes are weighed again to determine the weight loss and calculate the volume delivered. Pre- and post-weighing of syringes will also be accomplished with those used for dosing PYR, atropine, and 2-PAM. On every day of dosing, samples of the GD dosing solution used are taken and chemically analyzed by gas chromatography to confirm expected GD concentration.

Periodic observations, including signs of tremors, convulsions, salivation, prostration, and death, are recorded for 10 days. If all monkeys given a PYR dose predicted to produce a mean 23 percent peak AChE-I level do not survive for 48 hr following GD challenge and atropine/2-PAM therapy, the USAMRICD point of contact (POC) and the U.S. Army Contracting Officer's Representative (COR) will be notified before further research is conducted. If the predicted slope of the PYR dose-GD induced lethality response is low (< 1) such that reasonable estimation of a MED becomes difficult, further research will cease, the USAMRICD POC and the U.S. Army COR notified, and possible modifications of the experimental design discussed. This phase of experimentation will end when the standard error of the estimated i.m. PYR ED_{50} is less than 20 percent or when a maximum of 50 monkeys has been challenged.

(4) Efficacy of Oral PYR Pretreatment, at AChE-I Levels Similar to that Produced by the i.m. PYR MED, for a 5 X GD LD_{50} Challenge

Initially, in this phase, 8 monkeys will be fasted for 12 hr and then dosed with 1.2 mg/kg PYR via nasogastric intubation at eight hr intervals for a total of 4 doses, offering feed 4 hr after each PYR treatment. Blood samples will be taken prior to the first PYR dose, at 1.5 and 8 hr after each of the first 3 doses, and every 2 hr (or at different frequencies if early results indicate the desirability) after the 4th and final PYR dose until the RBC AChE activity is virtually normal. After a minimum 2 week washout period, the 8 monkeys, 4 at a time, will be given the same PYR dosing regimen, and when the RBC AChE-I level is predicted to be approximately the same as that found for the i.m. PYR MED, a blood sample will be taken and the monkey injected i.m. with 5 X GD LD_{50} and treated 1 min later with i.m. atropine/2-PAM.

Monkeys will be placed on a V-shaped platform, with arms and legs restrained, while being dosed with PYR, while blood samples are being taken, and when being dosed with GD and treated with atropine/2-PAM.

When the work of this phase has been accomplished, additional research will not be performed until the data have been compiled and analyzed and USAMRICD personnel have had an opportunity to review the results.

(5) Effect on the PYR MED of adding diazepam to the atropine/2-PAM treatment regimen

This phase of the study will be similar to phase 3. The PYR ED₅₀ and MED will be estimated in monkeys pretreated with PYR, challenged with 5 X GD LD₅₀, and treated 1 min later with atropine/2-PAM plus 0.1 mg/kg diazepam i.m. Assuming the PYR dose-lethal effect curve slope is unchanged from that estimated without diazepam in the post-GD therapy, as predicted in previous studies, no more than 30 monkeys should be needed to obtain a reasonable estimate of the effect of diazepam on therapy efficacy. This phase will end when the standard error of the estimated i.m. PYR ED₅₀ is less than 20 percent or when a maximum of 30 monkeys has been challenged.

Procedures used will be identical to that of phase 3 except that diazepam will be added to the therapy. Animals will be observed for 10 days with signs recorded.

(6) Protection from GD by PYR actions other than RBC AChE-I

If USAMRICD personnel are able to demonstrate efficacy of PYR pretreatment in guinea pigs against a GD challenge at 1, 4, or 24 hr after PYR-induced RBC AChE-I has returned to normal, then such a study may be repeated in monkeys. This would be accomplished using only a few monkeys at a time, starting at the earliest times after return to normal of RBC AChE activity, using atropine/2-PAM or atropine/2-PAM/diazepam therapy. Results of early stages of this phase would determine whether this study would be continued or would cease. No more than 16 monkeys would be used in this phase.

D. Study Preparations

Prior to challenge and i.m. dosing, hair over the anterior lateral aspect of the left thigh and over the posterior calf of the right leg are clipped using an Oster Model A-2 animal clipper with a No. 40 blade, or equivalent. Monkeys are weighed between 48 hr and 24 hr

prior to scheduled GD injection, using ketamine hydrochloride i.m. for chemical restraint, and doses of GD, pretreatment, and treatment compounds are administered on the basis of this body weight. Blood samples for baseline AChE activity may also be taken at this time.

E. Disposition of Experimental Animals

Monkeys on study are anesthetized with pentobarbital sodium, or other approved anesthetic solution, and perfused with a tissue fixative if, in the opinion of the Study Veterinarian or Study Director, conditions exist such that continuation on study would be inhumane. No monkey will be anesthetized within 24 hr of GD injection, however, since previous experience with this agent suggests that no reliable method exists to predict survivability during the acute phase of intoxication. A complete necropsy, with tissue harvesting, of all animals that die on study or animals that are anesthetized and perfused during the study or at the end of the 10 day holding period is accomplished, and animal remains are cremated. Tissue samples to be taken include: brain; spinal cord; dorsal root ganglia; peripheral nerves (i.e., brachial plexus, median [main trunk in upper arm and one muscular branch in lower arm where it enervates flexor carpi radialis muscle], phrenic [attached to diaphragm], and sciatic to include the main trunk, common peroneal branch overlying the lateral wing of the gastrocnemius muscle, and tibial branch just caudal to the bifurcation); eye; heart with aorta (1 inch); kidneys; liver; gall bladder; lung; spleen; pancreas; stomach; duodenum; jejunum; ileum; cecum; colon; abdominal skin; mesenteric lymph node; thyroids with parathyroids; testis; bone marrow of rib and femur; thymus; skeletal muscles (i.e., muscles innervated by collected peripheral nerves to include flexor carpi radialis, biceps brachii, diaphragm, intercostal, anterior tibialis, biceps femoris, and lateral head of the gastrocnemius); trachea; esophagus; parotid salivary gland; urinary bladder; pituitary gland; and adrenal glands. Tissues removed and preserved in formalin will be sent to USAMRICD for histopathologic evaluation.

12. Statistical Approach:

A modified up-down approach is used to estimate the untreated and atropine/2-PAM treated 48 hr GD LD₅₀s in this group of monkeys. Monkeys will be dosed with GD one or two at a time, starting at doses approximating 20 to 80 percent of the historic LD₅₀. If an animal dies at a given dose, the dose the next monkey receives, on a mg/kg body weight basis, is reduced, and conversely, if the first monkey lives, the next animal receives a higher dose. Based on historic information on the slope of the GD dose-lethal response curve and probit analysis of data as they are obtained, the best doses for challenging succeeding animals will be selected by the study director and study statistician in order to most

efficiently estimate the 48 hr GD LD₅₀s in the present population of monkeys. If, after a minimum of 4 monkeys have been challenged, the estimated LD₅₀ or PR falls within the 95 percent confidence limits of the Battelle historic Indian rhesus monkey 48 hr GD LD₅₀ or PR, that historic value will be accepted for the present population of monkeys.

A stagewise, adaptive dose allocation strategy is used to select PYR doses for phase 3 and phase 5 experiments. A GD dose 5 times the 48 hr LD₅₀ of untreated monkeys is used to assess the efficacy of PYR in preventing GD-induced lethality in monkeys treated with atropine/2-PAM or atropine/2-PAM/diazepam. Assuming that a PYR dose-GD induced lethality response exists, a stagewise designed experiment, using varied doses of PYR, is used to estimate the PYR dose-lethality response slope, the PYR ED₅₀, and the MED. In the first stage, two monkeys are tested at each PYR dose estimated in phase 2 (that dose producing 23 percent AChE-I and a dose 0.45 log units lower), and in the second stage, two monkeys are tested at each of two intermediary doses based on 0.15 log unit intervals. In subsequent stages, PYR doses are selected by the study statistician, in collaboration with the study director, based upon probit predictions of the PYR dose-lethality response slope and percentiles of response. As data are obtained, all information available is used to update estimates of the PYR dose-lethality response slope and estimated percentiles of response for allocating PYR doses to animals in succeeding stages of experimentation. After each stage, data will be closely monitored for the existence of a statistically significant PYR dose-lethality response relationship, and the i.m. PYR ED₅₀ and its standard error are estimated. Experiments will cease when the standard error of the estimated i.m. PYR ED₅₀ is less than 20 percent. This approach allows estimation of the PYR dose-lethality response relationship, PYR ED₅₀, and MED with a minimal number of animals.

13. Records to be Maintained:

- A. CSM accountability log and inventory
- B. Preparation of reagents, dose analyses and dosage administration
- C. Animal data
- D. Mortality data
- E. Clinical observations
- F. Decontamination, monitoring, and disposal records.

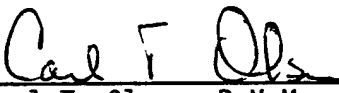
14. Reports:

- A. A draft final report is prepared within 30 days after completion of the exposures and analyses of the data. The draft final report includes:
 - (1) Signature page of key study personnel
 - (2) Experimental design
 - (3) Animal selection criteria and husbandry
 - (4) Test material description, analyses, preparation, and administration
 - (5) Clinical observations
 - (6) Statistical analyses of data
 - (7) Discussions and conclusions.
- B. Following receipt of draft final report comments from USAMRDC, a final report will be prepared within 30 days.

15. References:

- (1) Contract No. DAMD17-83-C-3129. Letter dated 25 July 1988, from Battelle to Commander, U.S. Army Medical Research Acquisition Activity, Ft. Detrick, MD, regarding results of Task 87-34: "The Effect of Treatment Regimens of Variable Concentrations of Atropine Sulfate in Combination with Pralidoxime Chloride on the Survival of Soman-Challenged Rhesus Monkeys Pretreated with Pyridostigmine Bromide".

16. Approval Signatures:



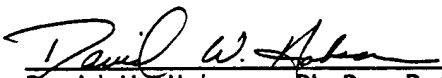
Carl T. Olson, D.V.M., Ph.D.
Study Director

22 October 1992
Date



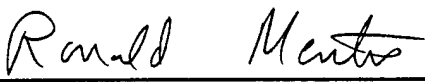
Robyn C. Kiser, B.S.
Study Supervisor

10-22-92
Date



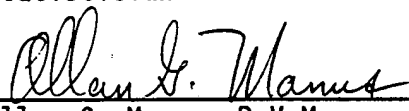
David W. Hobson, Ph.D., D.A.B.T.
MREF Principal Investigator and Manager

10-29-92
Date



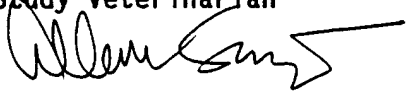
Ronald G. Menton, Ph.D.
Statistician

10-23-92
Date



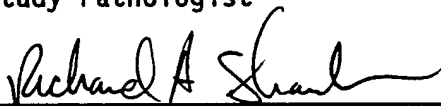
Allan G. Manus, D.V.M.
Study Veterinarian

10/23/92
Date



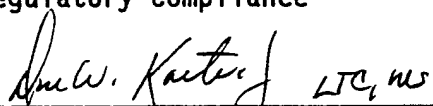
Allen W. Singer, D.V.M.
Study Pathologist

10-27-92
Date



Richard A. Shank, Manager
Regulatory Compliance

10.29.92
Date



LTC Don W. Korte, Jr., Ph.D.
USAMRICD COR

2 Dec 92
Date

Determination of the Minimum Effective Pyridostigmine
Pretreatment Dose in Monkeys Challenged with 5XLD₅₀
Soman and Treated with Atropine/2-PAM

Protocol Amendment No. 1

Change: Section 11. A (2) Initial Weight

To: Initial weights of monkeys provided for this study are 4 to more than 9 kg at the time of arrival at the MREF.

Change: Section 11. A (7) Acclimation

To: The protocol calls for monkeys be acclimated to restraint devices, either chairs or platforms where limbs are restrained with lanyards, prior to being placed on study or given a test compound. Due to the size of the monkeys provided by the U.S. Army for these studies, and concern for the safety of personnel handling these animals, some monkeys will not be acclimated to restraint boards prior to experimentation. These monkeys will be given a sedative (ketamine hydrochloride), removed from their cages and tied to restraint boards while body weights are obtained, blood samples taken, and hair clipped from injection sites. This operation will occur 24 to 48 hr prior to injecting test compounds. Monkeys selected for chairing while taking repetitive blood samples over 90 min following pyridostigmine injection, and monkeys to be dosed orally with pyridostigmine, will be acclimated to restraint without the use of sedation if this is deemed safe for monkeys and handlers. Animals to be dosed with an organophosphonate agent will not be sedated within 24 hr of injection. Some animals will be injected while restrained within their squeeze-back cage.

Approved By:

Carl T. Olson
Carl T. Olson, D.V.M., Ph.D.

2/8/93
Date

Don W. Korte, Jr. LTC, MS
Don W. Korte, Jr. COR

8 FEB 93
Date

Determination of the Minimum Effective Pyridostigmine
Pretreatment Dose in Monkeys Challenged with 5XLD₅₀
Soman and Treated with Atropine/2-PAM

Protocol Amendment No. 2

Change: Section 11. C (1), Initial Tests to Confirm LD₅₀s and Section 12,
Statistical Approach.

Replace the first sentence on page 6 which reads, "If, after 4 or more monkeys have been challenged, the estimated GD LD₅₀ in this study falls within the 95 percent confidence limits of the recent historic Battelle GD LD₅₀ in Indian rhesus monkeys, the historic LD₅₀ will be accepted for this group of animals." with "After a minimum of 4 monkeys has been challenged, the estimated LD₅₀ for this group of monkeys will be statistically compared to the Battelle historic LD₅₀ in rhesus monkeys. If the difference between the two LD₅₀s is determined to be statistically insignificant at the 5 percent level, the Battelle historic LD₅₀ will be updated and the updated value will be accepted for this group of animals". On page 11, replace the first sentence with the above change.

Reason for the Change:

Results of GD LD₅₀ testing after the use of 6 untreated monkeys indicate no significant difference between historic and present values, but the present estimated value is slightly below the 95 percent confidence limits of the historic value. Direct comparison to the 95 percent confidence limits of the historic LD₅₀ does not consider the variability in the LD₅₀ estimated for the current group of monkeys. The results obtained indicate no significant difference in LD₅₀ values, and the use of further animals to refine the LD₅₀ estimate is unjustifiable. Present results will be pooled with previous results to obtain a reasonable estimate of the GD LD₅₀ in the present population of animals.

Approved By:

Carl T. Olson
Carl T. Olson, D.V.M., Ph.D.

3/3/93
Date

Don W. Korte, Jr.
Don W. Korte, Jr., LTC, MS, USA, COR

3 MAR 93
Date

Determination of the Minimum Effective Pyridostigmine
Pretreatment Dose in Monkeys Challenged with 5XLD₅₀
Soman and Treated with Atropine/2-PAM

MREF Protocol 88, Amendment No. 3

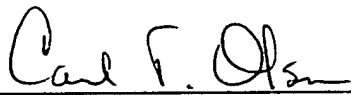
Change: Section 11.C.(3), Estimation of the i.m. PYR MED for a 5 X GD LD₅₀
Challenge

Replace the sentence on page 7 which reads, "Initially, using two monkeys at each PYR dose, monkeys will be treated with the PYR dose predicted to produce 23 percent AChE-I, and at three additional lower doses at 0.15 log unit intervals." with "Initially, using two monkeys at each PYR dose, monkeys will be treated with the PYR dose predicted to produce 23 percent AChE-I and a dose 0.45 log units lower."

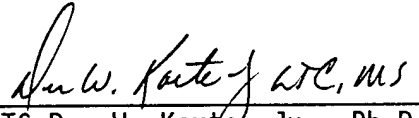
Reason for the Change:

Results of GD LD₅₀ testing, with and without atropine/2-PAM therapy, in Phase I of the study estimated the protective ratio of therapy at approximately three, which is significantly higher than results obtained in a previous study. Results of Phase II of the present study indicated a significant erythrocyte AChE-I at a PYR dose 0.45 log units lower than the PYR dose predicted to produce a 23 percent AChE-I. If deaths do not occur in monkeys challenged with 5 X GD LD₅₀ after pretreatment with a PYR dose 0.45 log units less than the PYR dose predicted to produce a 23 percent AChE-I and treated with atropine/2-PAM, the use of four monkeys at intermediate PYR doses is unjustified.

Approved By:


Carl T. Olson, D.V.M., Ph.D.
Study Director

3/30/93
Date


LTC Don W. Korte, Jr., Ph.D.
USAMRICD COR

31 MAR 93
Date

Determination of the Minimum Effective Pyridostigmine
Pretreatment Dose in Monkeys Challenged with 5XLD₅₀
Soman and Treated with Atropine/2-PAM

MREF Protocol 88, Amendment No. 4

Change: Replace 11.C.(4), Efficacy of Oral PYR Pretreatment, at AChE-I Levels Similar to that Produced by the i.m. PYR MED, for a 5 X GD LD₅₀ Challenge, with the following:

"11.C.(4), Efficacy of Oral PYR Pretreatment, at AChE-I Levels Similar to that Produced by an Effective i.m. PYR Dose, for a 5 X GD LD₅₀ Challenge

Initially, two monkeys previously acclimated to chair restraint and used in Phase II [11.C.(2)] will be given a dose of PYR syrup (Mestinox®) by intragastric intubation and AChE-I measured over time. Each of the two monkeys will be fasted overnight, and then restrained, a baseline blood sample obtained by femoral venipuncture, an oral feeding tube passed through a nostril and inserted to the level of the stomach, and a dose of PYR inserted in the tube and flushed from the feeding tube with approximately 5 mL of water. An intravenous catheter will be placed in a saphenous vein and taped in place, as in Phase II. Both monkeys will then be placed in restraint chairs and blood samples taken at approximately 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, and 180 min following PYR administration and analyzed for AChE-I. After taking the 180 min sample, monkeys will be returned to their cages, and if indicated, additional blood samples will be taken by femoral venipuncture. Results will give an indication of the maximum AChE-I attained, the time to maximum AChE-I, the rate of change in AChE-I, and the variability between animals. If the PYR dose initially selected is too great or too small to gain adequate information on its effect on RBC AChE-I, a different dose can be given to the same monkeys after a minimum 1-week washout. These monkeys can also be used later in Phase IV or in Phase V.

Following completion of the analysis of the data obtained, using a few animals per day, a total of 10 monkeys will be placed on restraint boards, have baseline blood samples taken, given a dose of PYR syrup intragastrically, and when AChE-I levels are between 5 and 10 percent, challenged with 5 X GD LD₅₀, and treated i.m. sequentially with 0.4 mg/kg atropine free base and 25.7 mg/kg 2-PAM starting at 1 min following the i.m. injection of GD. Monkeys given PYR intragastrically will be restrained at the time when the predicted AChE-I is 10 percent, a blood sample taken, and AChE-I analyzed. If the AChE-I is above 10 percent, another blood sample will be taken at a later time and analyzed for AChE-I. If AChE-I is

below 5 percent, that animal will be returned to his cage and not dosed with GD. After a minimum one week washout period, that animal can be dosed with PYR again and used in this phase of the study.

Monkeys dosed with GD and treated will be observed continuously for a minimum of 2 hr and at decreasing frequency thereafter for 10 days. Signs of GD intoxication, including tremors, convulsions, salivation/bronchial discharge, prostration, and death will be recorded. The 48-hr survival results for monkeys given an intragastric dose of PYR will be compared to the survival rate of monkeys given a i.m. dose of PYR which produces a similar AChE-I at the time of GD injection to determine whether a statistically significant difference exists."

Change: Replace 11.C.(5), Effect on the PYR MED of Adding Diazepam to the Atropine/2-PAM treatment Regimen, with the following:

"11.C.(5) Effect on the Efficacy of PYR Pretreatment of Adding Diazepam to the Atropine/2-PAM Treatment Regimen

The efficacy of various treatments in preventing GD-induced death will be evaluated by estimating the 48-hr median lethal dose of GD in monkeys receiving a given treatment. This will be accomplished in a manner similar to Phase I [11.C.(1)], using a modified up-and-down experimental design, challenging 1 or 2 monkeys per day for each treatment and increasing or decreasing the GD dose based on results obtained. Treatments to be evaluated are: 1) 0.4 mg/kg atropine(ATR)/25.7 mg/kg 2 PAM/0.1 mg/kg diazepam (DIAZ), with all treatments given i.m. sequentially at 1 min following challenge with various doses of GD; 2) PYR (4 μ g/kg) i.m. 45 min prior to GD challenge and ATR/2-PAM i.m. as in 1) above; and 3) PYR (4 μ g/kg) i.m. 45 min prior to GD challenge and ATR/2-PAM/DIAZ i.m. as in 1) above. Using chemical restraint, all monkeys will be weighed 24 to 48 hr prior to GD dosing and have injection sites clipped of hair and marked. At this time, blood samples will be taken by femoral venipuncture to determine a baseline AChE activity. Another blood sample will be taken just prior to GD injection to determine AChE-I level. Monkeys dosed with GD and treated will be observed continuously for a minimum of 2 hr and at decreasing frequency thereafter for 10 days. Signs of GD intoxication, including tremors, convulsions, salivation/bronchial discharge, prostration, and death will be recorded. The number of animals used in this phase will depend upon the results of 48-hr survival. If the standard error of the estimated 48-hr GD LD₅₀ following dosing of a minimum of five animals for any treatment regimen is less than 10 percent, testing of that treatment will cease. No more than 10 monkeys will be used for estimating the GD LD₅₀ for any treatment. Practical constraints in administering large doses of GD may limit the maximum GD dose administered to approximately 40 times the 48-hr GD LD₅₀ of untreated monkeys, as determined in Phase I [11.C.(1)]."

Change: 11.C.(6) Protection from GD by PYR Actions other than RBC AChE-I is deleted from this protocol.

11.E. Disposition of Experimental Animals

Add to the end of this paragraph the following sentence.

"All monkeys provided for this study that have not been injected with GD will be returned to USAMRICD following completion of the revised protocol."

12. Statistical Approach

Add the following sentence to the end of the first paragraph.

"The effect of adding PYR and/or diazepam to the atropine/2-PAM therapy regimen will be evaluated in a similar manner in Phase V [11.C.(5)], determining an estimate of the GD 48-hr LD₅₀ in an up-down manner using 5 to 10 monkeys in each treatment group."

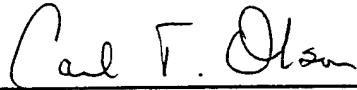
Change the first two sentences of the second paragraph to read as follows:

"A stagewise, adaptive dose allocation strategy is used to select PYR doses for phase III [11.C.(3)]. A GD dose 5 times the 48-hr LD₅₀ of untreated monkeys is used to assess the efficacy of PYR in preventing GD-induced lethality in monkeys treated with atropine/2-PAM."

Reasons for Changes:

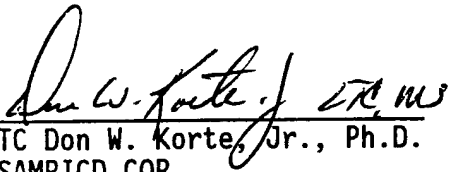
Results from Phase III [11.C.(3)] of this study demonstrated that i.m. doses of PYR of 4, 8.4 and 24 $\mu\text{g/kg}$ provide protection from 5XGD LD₅₀, as measured by 48-hr survival, that is statistically similar but greater than the protection provided by atropine/2-PAM alone. Since the AChE-I obtained with a PYR i.m. dose as low as 4 $\mu\text{g/kg}$ is on the order of 5 to 10 percent and that observed in monkeys given no PYR has been as high as 3.7 percent, determination of a PYR MED as proposed in this study is not practical. Therefore, Phase IV [11.C.(4)] has been changed to compare the pretreatment efficacy of an intragastric dose of PYR which produces 5 to 10 percent AChE-I with the 4 $\mu\text{g/kg}$ i.m. dose which produces a similar level of AChE-I. Since a PYR MED cannot be determined, the effect of adding diazepam to the treatment regimen cannot be determined as planned, and Phase V [11.C.(5)] has been changed to estimate the relative merit of various pretreatment/treatment regimens. Since research at USAMRICD was unable to demonstrate protection from GD in guinea pigs following the return to normal levels of RBC AChE activity after dosing with PYR, Phase VI [11.C.(6)] of this experiment has been deleted.

Approved By:



Carl T. Olson, D.V.M., Ph.D.
Study Director

6/18/93
Date



LTC Don W. Korte, Jr., Ph.D.
USAMRICD COR

21 Jun 93
Date

Determination of the Minimum Effective Pyridostigmine
Pretreatment Dose in Monkeys Challenged with 5XLD₅₀
Soman and Treated with Atropine/2-PAM

MREF Protocol 88, Amendment No. 5

Changes: Add to 11.C.(4), Efficacy of Oral PYR Pretreatment, at AChE-I Levels Similar to that Produced by an Effective i.m. PYR Dose, for a 5 X GD LD₅₀ Challenge, the following sentence, inserting it before the final sentence of the first paragraph of amendment 4.

"If the intragastric dose of the PYR syrup necessary to produce approximately 5 to 10 percent AChE-I within 2 hours of dosing is small such that the volume of the dose is too low to measure accurately, the Mestinon® will be diluted with deionized water (W/V) and the PYR concentration confirmed by chemical analysis."

Add to 11.C.(4), Efficacy of Oral PYR Pretreatment, at AChE-I Levels Similar to that Produced by an Effective i.m. PYR Dose, for a 5 X GD LD₅₀ Challenge, the following, inserting it after the final sentence of the second paragraph of amendment 4.

"If AChE-I is between 5 and 10 percent, a second blood sample will be taken at approximately 15 minutes or later and again analyzed for AChE-I. If the AChE-I is the same or less than measured in the previous sample, or if there is not more than a 5 percent increase in percent AChE-I, it will be assumed that the AChE-I level is constant or decreasing and the monkey will be injected with GD and treated with atropine and 2-PAM."

Reasons for Changes:

Phase IV was changed in amendment 4 to compare the pretreatment efficacy of an intragastric dose of PYR which produces 5 to 10 percent AChE-I with the 4 µg/kg i.m. dose which produces a similar level of AChE-I. Initial dosing of 2 monkeys with 125 µg/kg (approximately 60 µL of Mestinon®) intragastrically produced AChE-I in excess of 25 percent. In order to measure doses of PYR accurately for intragastric dosing to produce maximum levels of AChE-I on the order of 10 to 15 percent, it is necessary to dilute the PYR syrup. In order to be assured that the level of AChE-I is constant or decreasing rather than increasing at the time of GD dosing, it is necessary to obtain at least two blood samples for AChE-I analysis.

Approved By:

Carl T. Olson

Carl T. Olson, D.V.M., Ph.D.
Study Director

6/24/93
Date

Don W. Korte, Jr. LTC MS

LTC Don W. Korte, Jr., Ph.D.
USAMRICD COR

25 Jun 93
Date

Determination of the Minimum Effective Pyridostigmine Pretreatment Dose
in Monkeys Challenged with 5XLD₅₀ Soman and Treated with Atropine/2-PAM

MREF Protocol 88, Amendment No. 6

Change: Delete the second paragraph of 11.C.(4), Efficacy of Oral PYR Pretreatment, at AChE-I Levels Similar to that Produced by the i.m. PYR MED, for a 5 X GD LD₅₀ Challenge, as written in Amendment Nos. 4 and 5 and replace it with the following paragraph.

"Following completion of the analysis of the data obtained, using a few animals per day, a total of 10 monkeys will be placed on restraint boards, have baseline blood samples taken, and given an intragastric dose of PYR syrup predicted to produce, on the average, between 5 and 15 percent AChE-I at a given time following administration. At that time when average levels of AChE-I are predicted to be between 5 and 15 percent, monkeys will be challenged with 5 X the i.m. GD LD₅₀, and treated i.m. sequentially with 0.4 mg/kg atropine free base and 25.7 mg/kg 2-PAM starting at 1 min following the injection of GD. Just prior to GD challenge, approximately two blood samples will be taken by femoral venipuncture to determine AChE-I."

Reasons for Change:

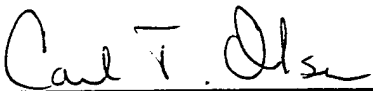
In the pharmacodynamic portion of the Phase IV studies, a great deal of variability in measured AChE-I was observed between animals, between different days of study for each animal, and in the analyses of blood samples. Based upon the results obtained in this first portion of Phase IV, implementation of the protocol for the second portion of this phase to compare the efficacy of PYR given intragastrically with doses of PYR given i.m. which produce approximately equivalent levels of AChE-I would be very difficult. The inter- and intra-animal variability, combined with the analytical variability, preclude administering an intragastric dose of PYR that consistently produces between 5 and 10 percent AChE-I in each animal.

In Phase III of this study, i.m. doses of PYR were administered and at a given time later the animal challenged with 5 X the 48-hr i.m. GD LD₅₀, with a blood sample taken approximately 5 min prior to GD challenge and analyzed for AChE-I. The inter-animal variability resulted in RBC AChE-I levels ranging from 6 to 18 percent when a PYR dose of 8.4 μ g/kg was administered i.m. Based on limited data collected in the first portion of Phase IV, the variability in

MREF Protocol 88
Medical Research and
Evaluation Facility
August 2, 1993

AChE-I for animals given PYR intragastrically is considerably greater. There were no significant differences in percent survival of Phase III monkeys challenged with GD following treatment with either of three i.m. PYR doses, even though mean percent AChE-I ranged from 6.9 percent at 4 μ g/kg PYR to 28.7 percent AChE-I at 24 μ g/kg PYR. Phase III results indicate that survival is not compromised by changes in AChE-I over a wide range, and statistical comparisons of the efficacy of PYR given by different routes of administration can be accomplished for doses producing approximately equivalent average levels of RBC AChE-I.

Approved By:



Carl T. Olson, D.V.M., Ph.D.
Study Director

8/2/93
Date



LTC Don W. Korte, Jr., Ph.D.
USAMRICD. COR

2 AUG 93
Date

Determination of the Minimum Effective Pyridostigmine Pretreatment
Dose in Monkeys Challenged with 5XLD₅₀ Soman and Treated
with Atropine/2-PAM

MREF Protocol 88, Amendment No. 7

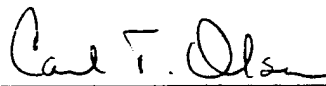
Change: Add to 11.C.(5). Effect on Efficacy of PYR Pretreatment of Adding Diazepam to the Atropine/2-PAM Treatment Regimen, as changed in Amendment No. 4, following the third sentence which lists the treatments to be evaluated, the following:

"Up to 10 additional monkeys may be used in a similar modified up-and-down fashion and treated with ATR/2-PAM 1 min following GD injection to attain a direct head-to-head comparison of this therapy regimen with the others listed above. If, after 4 or more monkeys have been treated with the ATR/2-PAM regimen, the estimated median lethal GD dose is not statistically different than that obtained in Phase I of this study, dosing of animals given this regimen will cease."

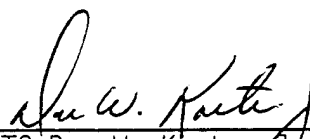
Reasons for Change:

After five monkeys have been injected with GD and given ATR/2-PAM/DIAZ treatment, the PR of this regimen appears to be less than that of treatment with ATR/2-PAM without DIAZ. Phase I monkeys were injected with GD and treated with ATR/2-PAM while restrained in a squeeze-back cage, and were not physically restrained on a tie-down board, nor were blood samples taken immediately prior to GD and therapy injections. This procedure was used because of the hazards to personnel posed by attempting to restrain the heaviest and strongest monkeys which were used in Phase I. This direct comparison is necessary to determine if the monkey size or the physical restraint affects the MLD and also, conversely, to determine if the addition of DIAZ to the treatment regimen adversely affects 48-hr survival.

Approved By:


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9/16/93
Date


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USAMRICD COR

16 Sep 93
Date

Determination of the Minimum Effective Pyridostigmine Pretreatment Dose
in Monkeys Challenged with 5XLD₅₀ Soman and Treated with Atropine/2-PAM

MREF Protocol 88, Amendment No. 8

Change: Add to 11.C.(5), Effect on Efficacy of PYR Pretreatment of Adding Diazepam to the Atropine/2-PAM Treatment Regimen, as changed in Amendment No. 7, following the changes of Amendment 7, the following:

"If, after 4 or more monkeys have been treated with ATR/2-PAM, the estimated median lethal GD dose is statistically different than that obtained in Phase I, but not different than that obtained for monkeys treated with ATR/2-PAM/DIAZ in Phase V, 5 additional monkeys may be challenged with GD and treated with ATR/2-PAM while restrained within their cages following procedures used in Phase I."

Reasons for Change:

The purpose of Phase V is to evaluate the relative efficacy of adding 0.1 mg/kg diazepam (DIAZ) to treatment regimen. Initially, GD dose-lethality response studies were planned for three treatment groups:

- PYR/ATR/2-PAM,
- PYR/ATR/2-PAM/DIAZ, and
- ATR/2-PAM/DIAZ.

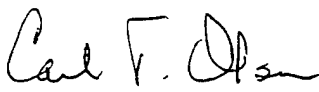
The GD dose-lethality response curves for untreated animals and animals treated with ATR/2-PAM were previously estimated in Phase I of this study. In Phase I, the GD MLD for animals treated with ATR/2-PAM was calculated to be 20.5 µg/kg with 95 percent confidence limits of 16.2 to 26.2 µg/kg. Early results in Phase V indicated that the GD MLD for animals treated with ATR/2-PAM/DIAZ may be substantially less than 20.5 µg/kg. To better understand this apparent difference in efficacy between animals treated with ATR/2-PAM and animals treated with ATR/2-PAM/DIAZ, the protocol was amended to include an ATR/2-PAM treatment group in Phase V. Concurrent testing of the two treatment groups, ATR/2-PAM and ATR/2-PAM/DIAZ, allowed us to determine that the apparent difference in the GD MLDs between the two treatments was not due to the addition of DIAZ to the treatment regimen. Phase V results to date suggest that there are no statistical differences between the GD MLDs estimated for animals treated with ATR/2-PAM and ATR/2-PAM/DIAZ, and that the GD MLDs for these two treatment groups are statistically less than that estimated for ATR/2-PAM in Phase I.

In Phase I, to minimize handling of the larger and more aggressive animals, monkeys were dosed while restrained in their cages. In Phase V, however, animals were removed from their cages, placed on slotted V-shaped platforms with limbs restrained, transported to a hood, and injected with GD and treatment before being returned to their cages. Because these different procedures may contribute to the apparent difference in GD MLDs estimated for animals treated with ATR/2-PAM in Phases I and V, the protocol has been amended.

Following completion of Phase V GD dose-lethality response experiments, the GD MLD for animals treated with ATR/2-PAM is estimated for these animals. Five animals are then injected, while restrained in their cages, with a fixed dose ($\mu\text{g/kg}$) of GD, the MLD for animals treated with ATR/2-PAM in Phase I. A one-tail hypothesis test is conducted to determine whether or not the number of survivors at 48 hr is incompatible, i.e., statistically different at the 5 percent significance level, with the GD dose-lethality response relation estimated for animals treated with ATR/2-PAM in Phase V.

These experiments should allow us to assess, using a minimal number of animals, whether or not the restraint procedures are partially responsible for the apparent difference in GD MLDs for animals treated with ATR/2-PAM in Phases I and V. Based on results to date, one or more survivors of the five dosed animals is incompatible with the GD MLD estimated for animals treated with ATR/2-PAM in Phase V. On the other hand, if the GD MLD estimated in Phase I for animals treated with ATR/2-PAM approximates the GD MLD for these five animals, then the probability of a result incompatible with the Phase V GD MLD is greater than 0.95.

Approved By:


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10/15/93
Date


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USAMRICD COR

15 OCT 93
Date

APPENDIX B

CHEMISTRY METHODS

ANALYSIS AND STRUCTURAL VERIFICATION OF PRALIDOXIME CHLORIDE

- A. Statement of Work: This method describes the procedures for verification of identity and quantitative measurement of pralidoxime chloride (2-PAM Cl) by high performance liquid chromatography (HPLC). The procedures for structural verification by proton nuclear magnetic resonance (NMR) of 2-PAM Cl present in drug formulations are also described. The HPLC effort can be conducted at either the MREF, HML or King Avenue site but NMR operations require the facilities at King Avenue.
- B. Materials To Be Used: The 2-PAM Cl used on this program will be provided by the U.S. Army Medical Research and Development Command (USAMRDC) or purchased from a traceable source. Upon receipt, the standard 2-PAM Cl will be stored in a desiccator at -10 to 10 degrees C or as directed by the supplier. The 2-PAM Cl aqueous solutions will be stored at 0-10 degrees C.

NMR spectra will be obtained on dilute solutions of the drug dissolved in > 99.8 percent deuterium oxide (Stohler Isotope Chemicals or equivalent). NMR tubes will be the Stohler Isotope Chemicals "Ultra Precision" model or the equivalent model from other manufacturers.

Other materials will include acetonitrile (spectroscopic grade or equivalent), deionized water or millipore water, glacial acetic acid, (Baker reagent grade or equivalent), tetrabutylammonium chloride (research grade or equivalent), benzophenone (research grade or equivalent), tetrabutylammonium nitrate (research grade or equivalent), sodium lauryl sulfate (dodecyl sulfate, sodium salt) (research grade or equivalent), and helium or nitrogen gas. All materials will be stored as directed by the supplier.

- C. Equipment: Proton NMR spectra will be obtained on Battelle's Varian 300 Mz. NMR spectrometer located at the King Avenue facility.

The HPLC analytical system, to be used consists of the following: HPLC pump, HPLC ultraviolet (UV) detector, HPLC injection system (autosampler), HPLC reverse-phase column, strip-chart recorder (optional), and electronic data system. Any equivalent system may be used once confirmation of performance has been established.

Other equipment includes: glass bottles, glass vials, Teflon® cap liners, microsyringes, pipettes, volumetric flasks, graduated cylinders, autosampler vials, refrigerator, Teflon® wash bottles, gas tight syringes, filter flask system, Pasteur pipettes, dropper bulbs, chart paper, spherisorb ODS 2 analytical HPLC column or equivalent, recorder pens, weighing paper, pipettes, pipette bulbs, and spatula.

- D. Sample Preparation: The drug formulation samples provided by the USAMRDC such as the 301 mg/mL dosing solutions are manipulated so that the interference of solvents and other components associated with the samples is minimized to provide relatively pure drug samples for NMR analysis.

HPLC analyses may be performed on either the dosing formulation as received, dilutions of the parent materials, or on reference standard solutions of known concentration.

1. Analytical Reference Standard: 2-PAM Cl solid reference standard supplied by the USAMRDC is dried at 100 C and ≤ 1.0 Torr for 3 hr prior to use. This is performed by placing the solid material contained in its original container which has had its cap removed into a pre-heated oven. The oven is sealed and the vacuum adjusted to ≤ 1.0 Torr. Store the dried reference material in a desiccator until use.
2. NMR Analysis: Approximately 2.0 mL of the 2-PAM Cl formulation is transferred to a 9.5 dram vial or a 50 or 100 mL round bottom 24/40 single neck flask and frozen therein by partially immersing in dry ice/acetone after the vial or flask is capped. This vial or flask is placed in (or attached to) a chamber of a lyophilization apparatus and subjected to high vacuum until the sample reaches a state of dryness.

NMR samples are prepared by dissolution of 30 mg of the dried samples in 0.75 mL deuterium oxide and are transferred to an NMR tube (tube capped after transfer) for NMR analysis.

3. HPLC Analysis: Samples are diluted with mobile phase so that the expected concentration range is between 0.01 and 0.10 mg/mL. Samples are refrigerated until analysis.

- E. Preparation of Standard Solutions: Standard solutions of 2-PAM Cl are prepared for an NMR reference spectrum and HPLC standard curve determinations.

1. NMR: Within a glove bag thoroughly flushed with dry nitrogen or argon, transfer approximately 50 mg of dried 2-PAM Cl standard into a 2 dram vial and cap this vial. Remove this vial from the glove bag and rapidly weigh 30 mg $\pm$ 0.1 mg of the contained material into a second 2 dram vial. Dissolve the sample in 0.75 mL of deuterium oxide and recap the bottle to minimize the contamination of the sample with undeuterated moisture. Transfer this solution with a Pasteur pipet to an NMR tube (tube capped after transfer) for NMR analysis. Return the non-used standard material to a desiccator.
2. HPLC: Accurately weigh 50 mg $\pm$ 0.1 mg of 2-PAM Cl onto weighing paper. Quantitatively transfer the 2-PAM Cl into a 50-mL volumetric flask containing approximately 40 mL of mobile phase (see Section F.2). Mix the solution thoroughly. Dilute to 50 mL with

water and remix the solution. The resulting concentration of the 2-PAM C1 stock will be approximately 1 mg/mL.

Weigh out $10 \text{ g} \pm 0.1 \text{ g}$ of benzophenone, the internal standard (IS), and quantitatively transfer the material into a 25-mL volumetric flask containing approximately 20 mL of acetonitrile. Mix well until dissolved. Dilute to 25.0 mL with acetonitrile and remix the solution.

The resulting concentration of the benzophenone internal standard stock is 400 mg/mL.

Mix and dilute the 2-PAM C1 stock solution with mobile phase (see Section F.2) in 10 mL volumetric flask as follows:

1.0-mL stock + 9.0-mL mobile phase
0.50-mL stock + 9.5-mL mobile phase
0.25-mL stock + 9.75-mL mobile phase
0.10-mL stock + 9.90-mL mobile phase
0.0-mL stock + 10.0-mL mobile phase

After the standards have been prepared, each level is then spiked 5 μL of the internal standard solution. The final standard concentrations are 0.10, 0.050, 0.025, 0.010, and 0.0 mg per mL.

Diluted standard solutions are kept refrigerated until used. Standards may be stored refrigerated for up to 30 days.

F. Analysis Start-Up: NMR is performed to verify the structure of the 2-PAM C1. HPLC is performed to quantitatively determine the concentration of 2-PAM in the samples and identity confirmation of 2-PAM in the dosing solution by retention indices comparison.

1. NMR: Calibrate the NMR instrument and data system according to Battelle's Commercial and Industrial Technology Division's, Good Manufacturing Practices (GMP) SOP III-008 entitled, "Operation and Maintenance of NMR Spectrometer".
2. Quantitative HPLC: Prepare HPLC mobile phase buffer for quantitative analysis by dissolving 2.7 g of tetramethylammonium chloride in approximately 900 mL of deionized water. Add 1.0 mL of glacial acetic acid and dilute to 1 L and mix. Store in a clean, 1-L glass bottle. Use within 30 days.

The mobile phase may be established using a gradient system with a 40 percent buffer:60 percent acetonitrile ratio or mixed prior to analysis. To mix the mobile prior to analysis, add 400 mL of the buffer prepared above to a 1-L glass bottle and add 600 mL of

acetonitrile and mix. Once the buffer has been prepared, it must be filtered and used within 30 days.

Insure the appropriate analytical column has been installed in the analytical system and that the injector is equipped with at least a 20- μ L sample injection loop.

All mobile phase must be degassed for at least 5 min with nitrogen, or helium prior to use.

The detector and the pump must be turned on for a warm-up period of at least 15 min prior to system evaluation. The pump flow must be set at 1.2 mL/min during the warm-up period. After approximately 15 min, measure the flow for 5 min with a 10-mL graduated cylinder. The flow rate must be set at 1.2 ± 0.1 mL/min. Adjust the flow rate setting on the pump if necessary to obtain an actual flow rate within these limits and re-check.

After the pump has been on for about 30 min, adjust the detector zero per the operator's manual. Adjust the recorder to electrical zero at "0" chart units. Adjust the detector zero to slightly above the electrical zero position with the recorder balance control.

3. HPLC Identity Confirmation: Prepare HPLC mobile phase buffer for the initial identity confirmation using a Supelco LC-1 column or equivalent by dissolving 6.0 g of sodium lauryl sulfate and 1.0 g of tetrabutylammonium nitrate in 1,000 mL of deionized water. Add 20 mL of glacial acetic acid to the solution and mix. Filter the solution with a 5 μ m filter and store in a clean glass bottle. Use within 30 days.

The mobile phase may be established using a gradient system with a 60 percent buffer:40 percent acetonitrile ratio or mixed prior to analysis. To mix the mobile prior to analysis, add 600 mL of the buffer prepared above to a 1-L glass bottle and add 400 mL of acetonitrile and mix. Once the buffer has been prepared, it must be used within 30 days.

Insure the appropriate analytical column has been connected to the injector and detector, and that the injector is equipped with a 20- μ L sample injection loop.

All mobile phase must be degassed for at least 5 min with nitrogen, or helium prior to use.

The detector and the pump must be turned on for a warm-up period of at least 15 min prior to system evaluation. The pump flow must be set at 1.0 mL/min during the warm-up period. After approximately 15 min, measure the flow for 5 min with a 10-mL graduated cylinder. The flow

rate must be set at 1.0 ± 0.1 mL/min. Adjust the flow rate setting on the pump if necessary to obtain an actual flow rate within these limits and re-check.

After the pump has been on for about 30 min, adjust the detector zero per the operator's manual. Adjust the recorder to electrical zero at "0" chart units. Adjust the detector zero to slightly above the electrical zero position with the recorder balance control.

- G. Analysis of Samples: NMR is performed for structural confirmation. HPLC standards and collected samples are analyzed to determine concentration and identify confirmation.

1. NMR: Multiple acquisitions (≥ 150 transients) are generally required. Spectra will be printed on standard NMR paper and computer referenced to the chemical shift of sodium 2,2-dimethyl-2-silapentane-5-sulfonate contained in the deuterium oxide. A listing of shifts and parameters used will be obtained.
2. Quantitative HPLC: The following is a set of HPLC conditions that have been found to be satisfactory for quantitative analysis of 2-PAM C1:

Column: Alltech Spherisorb-ODS 2 or equivalent and Supelco LC-18 Guard Column or equivalent.

Mobile Phase: See Section F.2.

Detector: UV @ 298 nm

Flow Rate: 1.2 mL/min

Injection Volume: 20 μ L

For quantitative analysis of 2-PAM C1 samples, transfer 1-mL duplicate aliquots of each 2-PAM C1 standard to autosampler vials and place the vials in the autosampler in ascending concentration order. Set up the data system to acquire data for each standard as described in the data system instruction manual. Transfer 1-mL duplicate aliquots of each sample to autosampler vials and place the vials in the autosampler.

For every ten samples to be analyzed, at least one blank sample and one standard must be analyzed. All samples must be analyzed under the same conditions used for the standards.

3. HPLC Identity Confirmation: For confirmation of the identity of 2-PAM C1 by HPLC, a second set of HPLC conditions is employed. The following is a set of HPLC conditions found to be satisfactory for the confirmation of 2-PAM C1:

Column: Supelco LC-1 or equivalent 250 x 4.6 mm, 5 micron and Supelco LC-1 guard column or equivalent.

Mobile Phase: See Section F.3

Detector: UV @ 254 nm

Flow Rate: 1.0 mL/min

Injection Volume: 20 μ L

For confirmation purposes, analyze a 2-PAM C1 standard and a formulation sample under these HPLC conditions.

H. HPLC Instrument Shut-Down:

1. When the instrument is not to be used for extended periods of time, the system must be shut down following manufacturer's instructions to ensure column life and instrument stability.
2. For overnight shut-down, turn off the UV detector, chart recorder, and pump controller.
3. For weekend shut-down, follow the same procedure as for overnight shut-down but also cap off the analytical column to prevent the solid phase from drying.

I. Data Reduction: The NMR spectrum obtained in Section G.1 is compared with the reference spectrum to verify structural identity. HPLC samples analyzed in Section G.2 are compared with results obtained from standards to determine concentration.

1. NMR: Compare the NMR spectrum for the sample with the spectrum obtained for the 2-PAM C1 reference standard. Verify correspondence of chemical shifts, multiplicities, and intensities for structural verification in conjunction with HPLC findings.
2. Quantitative HPLC: Obtain printouts of the peak area ratios for each standard and sample as described in the instruction manual. Prepare a standard curve from the peak area ratios versus concentration of the standards.

Determine the 2-PAM C1 concentration in the samples and control standards using the standard curve. If necessary, correct for any dilution made to the samples prior to analysis.

If the response for any of the control standards varies from the predicted response by more than ± 10 percent, then the samples associated with that standard are reanalyzed.

3. HPLC Identity Confirmation: Compare the retention times and relative responses of the 2-PAM Cl standard and sample peak for structural confirmation.

Originated by: Timothy L. Hayes 1-28-93
Timothy L. Hayes, B.A. Date
Principal Research Scientist

Reviewed by: Herman Benecke 1/29/93
Herman Benecke, Ph.D. Date
Senior Research Scientist

METHOD FOR THE ANALYSIS AND STRUCTURAL VERIFICATION OF
ATROPINE BASE IN CITRATE BUFFER

- A. Statement of Work: This method describes the entire procedures for verification of identity and quantitative measurement of atropine free base by high performance liquid chromatography (HPLC). The procedures for structural verification by nuclear magnetic resonance (NMR) of atropine present in drug formulations are also described. The HPLC effort can be conducted at either the MREF, HMRF or King Avenue site. The NMR operations require facilities only available at King Avenue.

B. Materials To Be Used:

Solvents and Chemicals: The atropine sulfate solid which will be used on this program for preparation of analytical standards will be provided by the U.S. Army Medical Research and Development Command (USAMRDC) or a commercial supplier that can provide an established purity.

If the atropine dosing solution is not received in a pre-packaged form with specified storage conditions, upon receipt the atropine dosing solution will be stored in subdued lighting at -10 to 10 degrees C. If a pre-packaged form has been received, it will be stored as directed by the supplier. The atropine concentration in the prepackaged formulation is typically 2.85 mg/mL.

NMR spectra will be obtained on dilute solutions of the drug dissolved in > 99.8 percent deuterium oxide (Stohler Isotope Chemicals or equivalent). NMR tubes will be the Stohler Isotope Chemicals "Ultra Precision" model or the equivalent model from other manufacturers. Deuterated sulfuric acid in deuterium oxide (98 wt. percent solution; 99.5+ percent deuterium) from Aldrich Chemical Company (or equivalent) will be used to convert atropine free base to its sulfate.

Other materials will include color pHast paper (EM science) acetonitrile (spectroscopic grade or equivalent), methanol (spectroscopic grade or equivalent), benzene (spectroscopic grade or equivalent), deionized water or millipore water, glacial acetic acid (reagent grade or equivalent), tetrabutylammonium chloride (98+ percent or equivalent), sodium lauryl sulfate (98 percent or equivalent), sodium heptane sulfonate (1-heptane sulfonic acid, sodium salt) (98+ percent or equivalent), tetramethylammonium chloride (98+ percent or equivalent), and helium or nitrogen gas.

- C. Equipment: Freezer, refrigerator, labels, absorbent paper, squirt bottles, wiping tissues, beakers, bottles, maxi-vials, pipettes, pipette bulbs, laboratory coat, protective eyewear, spatula, syringes, needles, forceps, and latex gloves.

Proton NMR spectra will be obtained on Battelle's Varian 300 Mz NMR spectrometer located at the King Avenue facility.

The HPLC analytical system necessary for this operation shall have the following components: HPLC pump, HPLC ultra violet (UV) detector, HPLC injection system (autosampler), analytical column, strip-chart recorder (optional), electronic data system. Any equivalent system may be used once confirmation of performance has been established.

D. Procedures:

1. Sample Preparation: The drug formulation samples provided for analysis will be manipulated so that the interference of solvents and other components associated with the samples is minimized to provide relatively pure drug samples for NMR analysis.

HPLC analyses may be performed on either the dosing formulations as received, dilutions of the parent materials, or on reference standard solutions of known concentration.

- a. Analytical Reference Standard: Solid atropine sulfate standard used as a reference material is dried in a vacuum oven at 100 C, and <1.0 Torr for 3 hours prior to use. This is performed by placing the solid material contained in its original container (without cap) into a pre-heated oven. Store the dried standard material in a desiccator until use.
- b. NMR: For the NMR sample preparation, 12 mL of test sample is made basic with 24 mL of 0.1 M sodium hydroxide to reach a pH of approximately 13 (verified by color pHast paper). This solution is stirred rapidly with benzene (60 mL) using a magnetic stir bar for 15 min and then poured through phase separation paper (with 12 mL benzene rinse). The filtrate is stirred rapidly for 1 min with 24 mL deionized water using a magnetic stir bar and this mixture is passed again through a fresh phase separation paper (with 12 mL benzene rinse). The benzene filtrate is evaporated in a rotary evaporator (in the hood) to yield atropine as its free base. The material is dried one hour at ambient temperature at ≤ 1.0 Torr in a vacuum oven. The sample is weighed and 25-30 mg removed for determining its NMR spectrum. The sulfate is reformed by adding a slight molar excess of dilute D_2SO_4 in D_2O to the free base with a total volume of 0.75 mL.

NMR samples are prepared by transfer of the deuterium oxide solution into an NMR tube (tube capped after transfer) for NMR analysis.

- c. HPLC Analysis: Samples are either analyzed directly or can be diluted so that the expected concentration range is between 0.1 and 1.0 mg/mL.
2. Preparation of Standard Solutions: Standard solutions of atropine sulfate are prepared for NMR reference spectrum and HPLC standard curve determinations.
- a. NMR: Within a glove bag thoroughly flushed with dry nitrogen or argon, transfer approximately 50 mg of atropine sulfate into a 2 dram vial and cap this vial. Remove this vial from the glove bag and rapidly weigh $25-30 \pm 0.1$ mg of the contained material into a second 2 dram vial. Dissolve the sample in an accurately measured volume of 0.75 mL of deuterium oxide in a 1 or 2 dram vial and recap the vial to minimize the contamination of the sample with undeuterated moisture. Transfer this solution with a Pasteur pipet to an NMR tube (tube capped after transfer) for NMR analysis. Return the non-used standard material to a desiccator.
 - b. HPLC: Weigh 50 ± 0.1 mg of atropine sulfate onto weighing paper. Quantitatively, transfer the sample into a 50-mL volumetric flask containing approximately 40 mL of mobile phase. Mix the solution thoroughly on a vortex mixer. Dilute to 50.0 mL with the mobile phase and remix the solution. The resulting concentration of the atropine sulfate will be approximately 1 mg/mL.

Mix and dilute the atropine sulfate stock solution with the mobile phase as follows:

10.0-mL stock +	0.0-mL mobile phase
5.0-mL stock +	5.0-mL mobile phase
2.5-mL stock +	7.5-mL mobile phase
1.0-mL stock +	9.0-mL mobile phase
0.0-mL stock +	10.0-mL mobile phase

The atropine sulfate concentrations obtained are 1.00, 0.50, 0.25, 0.10, and 0.0 mg per mL.

Diluted standard solutions are kept refrigerated until use. Standards may be kept refrigerated for up to 30 days.

3. Analysis Start-Up: NMR is performed to verify the structure of atropine sulfate. HPLC is performed to quantitatively determine the concentration of atropine sulfate and confirm the identity of the atropine in the samples.
- a. NMR: Calibrate the NMR instrument and data system according to Battelle's Commercial and Industrial Technology Division's, Good

Manufacturing Practices (GMP) SOP III-008 entitled, "Operation and Maintenance of NMR Spectrometer".

- b. Quantitative HPLC: Prepare HPLC mobile phase for quantitative analysis by dissolving 2.2 g of sodium heptane sulfonate (1-heptane sulfonic acid sodium salt) and 2.7 g of tetramethylammonium chloride in approximately 90 mL of deionized water. Add 1.0 mL of glacial acetic acid and dilute to 1 L and mix. Filter buffer solution before using.

The mobile phase may be established using a gradient system with a 78 percent buffer:2 percent methanol:20 percent acetonitrile ratio or mixed prior to analysis. To mix the mobile prior to analysis, add 780 mL of the buffer prepared above to a 1-L glass bottle, add 20 mL of methanol and 200 mL of acetonitrile and mix. Once the buffer has been prepared, it must be filtered and used within 30 days.

Insure that the appropriate analytical column has been installed in the analytical system, and that the injector is equipped with at least a 20- μ L sample injection loop.

All mobile phase must be filtered and degassed for at least 5 min with nitrogen or helium, prior to use.

The detector and the pump must be turned on for a warm-up period of at least 15 min prior to system evaluation. The pump flow must be set at 1.0 mL/min during the warm-up period. After approximately 15 min, measure the flow for 5 min with a 10-mL graduated cylinder. The flow rate must be set at 1.0 ± 0.1 mL/min. Adjust the flow rate setting on the pump controller if necessary to obtain an actual flow rate within these limits and re-check flow.

After the pump has been on for 30 min, adjust the detector zero with the detector attenuation set at the appropriate attenuation. Adjust the recorder to electrical zero at "0" chart units. Adjust the detector zero to slightly above the electrical zero position with the recorder balance control.

- c. HPLC Identity Confirmation: Prepare HPLC mobile phase for identity confirmation by adding 6.0 g of sodium lauryl sulfate and 1.0 g of tetrabutylammonium nitrate to a 1-L volumetric flask and dissolve the reagents in approximately 500 mL of deionized water. Add 20 mL of glacial acetic acid to the solution and mix. The volumetric flask is filled to the 1-L mark and the solution re-mixed. Filter the solution with a 5- μ m filter and store in a clean glass bottle. Use within 30 days.

The mobile phase may be established using a gradient system with a 60 percent buffer:40 percent acetonitrile ratio or mixed prior to analysis. To mix the mobile prior to analysis, add 600 mL of the buffer prepared above to a 1-L glass bottle and add 400 mL of acetonitrile and mix. Once the buffer has been prepared it must be used within 30 days.

Insure that a Supelco LC-1 column or equivalent has been connected to the injector and detector and the injector is equipped with a 20- μ L sample injection loop.

All mobile phase must be degassed for at least 5 min with helium or nitrogen prior to use.

The detector and the pump must be turned on for a warm-up period of at least 15 min prior to system evaluation. The pump flow must be set at 1.0 mL/min during the warm-up period. After approximately 15 min, measure the flow for 5 min with a 10-mL graduated cylinder. The flow rate should be 1.0 ± 0.1 mL/min. Adjust the flow rate setting on the pump if necessary to obtain an actual flow rate within these limits and re-check.

After the pump has been on for 30 min, adjust the detector zero with the detector set at the appropriate attenuation. Adjust the recorder to electrical zero at "0" chart units. Adjust the detector zero to slightly above the electrical zero position with the recorder balance control.

4. Analysis of Samples: NMR is performed for structural confirmation. HPLC standards and collected samples are analyzed to determine concentration and identity confirmation.
 - a. NMR: Multiple acquisitions (≥ 150 transients) are generally required. Spectra will be printed on standard NMR paper and computer referenced to the chemical shift of sodium 2,2-dimethyl-2-silapentane-5-sulfonate contained in the deuterium oxide. A listing of shifts and parameters used will be obtained.
 - b. Quantitative HPLC: The following is a set of HPLC conditions that have been found to be satisfactory for quantitative analysis of atropine sulfate by HPLC (See Reference 1):

Column: C18 μ -Bondapak or equivalent, 250-mm long x 4.6-mm inner diameter with 5 micron particle size.

Mobile Phase: See Section D.3.b

Detector: UV @ 260 nm

Flow Rate: 1.8 mL/min

Injection Volume: 20 μ L

For quantitative analysis of atropine sulfate samples, transfer 1-mL duplicate aliquots of each atropine sulfate standard to autosampler vials and place the vials in the autosampler in ascending concentration order. Set up the data system to acquire data for each standard as described in the instruction manual. Transfer 1-mL duplicate aliquots of each sample to autosampler vials and place the vials in the autosampler.

For every ten samples to be analyzed, one blank sample and one standard must be analyzed as a minimum. All samples must be analyzed under the same conditions as used for the standards.

- c. HPLC Identity Confirmation: For confirmation of the identity of atropine sulfate by HPLC, a second set of HPLC conditions is employed. The following is a set of HPLC conditions found to be satisfactory for the confirmation of atropine.

Column: Supelco LC-1, 250-mm long x 4.6-mm inner diameter, with 5 micron particle size.

Mobile Phase: See Section D.3.c

Detector: UV @ 254 nm

Flow Rate: 1 mL/min

Injection Volume: 20 μ L

For confirmation purposes, analyze an atropine sulfate standard and a sample from the formulation under these HPLC conditions.

5. HPLC Instrument Shut-Down:

- a. When the instrument is not to be used for extended periods of time, the system must be shut down following manufacturer's instructions to ensure column life and instrument stability.
- b. For overnight shut-down, turn off the UV detector, chart recorder, and pump controller.
- c. For weekend shut-down, follow the same procedure as for overnight shut-down but also cap off the analytical column to prevent the solid phase from drying.

6. Data Reduction: The NMR spectra obtained in Section D.4 are compared to reference NMR spectra for atropine to verify structural identity. The HPLC samples analyzed in Section D.4 are compared with results obtained from known reference standards to determine concentration.
- NMR: Compare the NMR spectrum for the sample with the spectrum obtained for the atropine sulfate reference standard. Verify correspondence of chemical shifts, multiplicities, and intensities for structural verification in conjunction with HPLC findings.
 - Quantitative HPLC: Obtain printouts of the peak areas for each standard and sample as described in the data system instruction manual. Prepare a standard curve from the peak areas versus concentration of the standards.

Determine the atropine sulfate concentration in the samples and control standards using the standard curve. If necessary, correct any dilution made to the samples prior to analysis.

If the response for any of the control standards varies from the predicted response by more than ± 10 percent, then the samples associated with that standard are reanalyzed.

- HPLC Identity Confirmation: HPLC confirmation of the identity of atropine sulfate is performed by analysis under a second set of HPLC conditions. Compare the retention times and relative responses of the atropine sulfate reference standard and sample peak for structural confirmation in conjunction with the first set of HPLC results and NMR conclusions.

E. Reference:

- "Assay of Formulated Atropine Solution, WR-6241AK, B107753, Lot No. RU7144," Report No. 527, Contract No. DAMD17-85-C-5141, SRI International Project No. 8504, December 10, 1985.

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**METHOD FOR THE ANALYSIS AND STRUCTURAL VERIFICATION
OF PYRIDOSTIGMINE BROMIDE**

- A. Statement of Work: The purpose of this work is to verify the structural identity of pyridostigmine bromide and to analyze quantitatively for the amount of pyridostigmine bromide present in drug formulations.

B. Materials To Be Used:

Solvents and Chemicals: Pyridostigmine bromide - Prior to analysis, reference pyridostigmine bromide and its solutions will be stored in subdued lighting at room temperature.

Formulated pyridostigmine bromide will either be in the form of an aqueous injectable solution (1 mg/mL) or in Mestinon syrup (12 mg/mL). Proton nuclear magnetic resonance (NMR) spectra will be obtained on dilute solutions of the drug dissolved in > 99.8 percent deuterium oxide (Stohler Isotope Chemicals or equivalent). NMR tubes will be the Stohler Isotope Chemicals "Ultra Precision" model or the equivalent model from other manufacturers.

Other materials will include acetonitrile (spectroscopic grade or equivalent), deionized water or Millipore water, glacial acetic acid (reagent grade or equivalent), tetrabutylammonium chloride (98+ percent or equivalent), tetrabutylammonium nitrate (99 percent or equivalent), sodium lauryl sulfate (98 percent or equivalent), p-aminobenzoic acid (99 percent or equivalent), hydrobromic acid (48 percent reagent grade or equivalent), Amberlite® IR-120 (plus) ion exchange resin or equivalent, and helium or nitrogen gas.

- C. Equipment: Proton NMR spectra will be obtained on Battelle's Varian 300 MHz NMR spectrometer located at the King Avenue facility.

The HPLC analytical system to be used consists of the following: HPLC pump, HPLC ultraviolet (UV) detector, HPLC autosampler, analytical column, strip-chart recorder, and electronic data system.

Other equipment includes glass bottles, labels, HPLC mobile phase filter system, wiping tissues, beakers, pipette bulbs, spatula, forceps, weighing paper, glass vials, Teflon® cap liners, microsyringes, pipettes, volumetric flasks, graduated cylinders, autosampler vials, refrigerator, pH meter, Teflon® wash bottles, Eppendorf pipettor, pipettor tips, Pasteur pipettes, chart paper, and recorder pens.

D. Procedures:

1. Sample Preparation: The drug formulation samples provided for analysis will be manipulated so that the interference of solvents and

other components associated with the samples is minimized to provide relatively pure drug samples for NMR analysis.

HPLC analyses may be performed on either the dosing formulations as received, dilutions of the parent materials, or on reference standard solutions of known concentration. All sample preparation will be conducted in a hood.

- a. Analytical Reference Standard: Pyridostigmine bromide solid reference standard is dried over P_2O_5 at 100 C and ≤ 1.0 Torr for 4 hr prior to use in a vacuum oven. This is performed by placing the solid material contained in its original container which has had its cap removed into a preheated oven. The oven is sealed and the vacuum adjusted to ≤ 1.0 Torr. Store the dried standard material in a desiccator until use.
- b. NMR: For the NMR sample preparation from Mestinon syrup, 2.0 mL of the syrup is dissolved in 48 mL of water and the solution slowly passed through a cation-exchange resin bed (Amberlite® IR-120 (plus) ion exchange resin, 1 x 4.5 cm). The column is washed with 50 mL of deionized water and the pyridostigmine bromide eluted with 200 mL of 1 N HBr prepared by diluting 22.6 mL of 48 percent HBr with 177.4 mL of deionized water. The eluate is evaporated to dryness under reduced pressure at 50 C in a rotary evaporator using a water aspirator. The sample is then dried one hour in a vacuum oven at ambient temperature at < 1.0 Torr.

NMR samples are prepared to be 15-25 mg/mL concentration by dissolving the entire sample (which is weighed to the nearest 0.1 mg) in 1.0 mL deuterium oxide. Transfer 0.75 mL solution into an NMR tube after filtration through a small cotton plug (in a Pasteur pipet) to remove any visible particulate. Cap tube after transfer.

For the NMR sample preparation from aqueous injectable solutions, 25 mL of the solution is transferred to a 250-mL round bottom 24/40 single neck flask. The flask is stoppered and the contents are frozen therein by partially immersing in dry ice/acetone and spinning the flask to obtain a thin shell. This flask is attached to a lyophilization chamber and subjected to high vacuum until the sample reaches a state of dryness.

NMR samples are prepared to be approximately 15-25 mg/mL by weighing 11-19 mg of freeze dried material into a 1 or 2 dram vial. Add 0.75 mL of deuterium oxide and efficiently transfer the complete solution to an NMR tube (tube capped after transfer) with a Pasteur pipet.

- c. HPLC: Samples are diluted with deionized water so that the deionized water range is between 0.02 and 0.08 mg/mL.
2. Preparation of Standard Solutions: Standard solutions of pyridostigmine bromide are prepared for NMR reference spectrum and HPLC standard curve determination.
- a. NMR: Accurately weigh to within 0.1 mg 15 mg of pyridostigmine bromide reference standard. Transfer the sample into a 1 or 2 dram screw-capped vial and close tightly. Dissolve the sample in 0.75 mL of deuterium oxide and recap the vial to minimize the contamination of the sample with undeuterated moisture. Transfer this solution with a Pasteur pipet to an NMR tube (tube capped after transfer) for NMR analysis. Return the non-used standard material to a desiccator.
- b. HPLC:
- Pyridostigmine Bromide Stock Solution: Accurately weigh to within 0.1 mg 50 mg of pyridostigmine bromide. Dissolve the sample in approximately 40 mL of deionized water. Dilute to 50.0 mL with deionized water.
- Internal Standard Stock Solution: Accurately weigh to within 0.1 mg 10 mg of p-aminobenzoic acid, the internal standard (IS), and dissolve in approximately 40 mL of methanol. Dilute to 100 mL with methanol.
- Mix and dilute the pyridostigmine bromide stock solution with deionized water as follows:
- | | |
|---------------|-----------------|
| 1.0-mL stock | + 4.0-mL water |
| 0.50-mL stock | + 4.5-mL water |
| 0.25-mL stock | + 4.75-mL water |
| 0.10-mL stock | + 4.90-mL water |
| 0.0-mL stock | + 5.0-mL water |
- Working standards are prepared by diluting 1.0-mL aliquots of each of these pyridostigmine bromide solutions with 1.0-mL aliquots of IS solution to give the following pyridostigmine bromide concentrations of 0.10, 0.050, 0.025, 0.010, and 0.0 mg/mL.
- Diluted standard solutions are kept refrigerated until use. Standards may be kept refrigerated for up to 30 days.
3. Analysis Start Up: NMR is performed to verify the structure of pyridostigmine bromide. HPLC is performed to quantitatively determine the concentration of pyridostigmine bromide in the samples.

- a. NMR: NMR analysis is carried out after the sample being analyzed is placed in the magnet and the response for the particular sample has been maximized. Follow the procedures described in Battelle's Commercial and Industrial Technology Division's, Good Manufacturing Practices (GMP) SOP III-008 entitled, "Operation and Maintenance of NMR Spectrometer".
- b. Quantitative HPLC: Prepare HPLC mobile phase for quantitative analysis by dissolving 3.2 g of tetramethylammonium chloride and 6.9 g of KH_2PO_4 in approximately 900 mL of deionized water. Dilute to 1 L and mix. Adjust the pH of the solution to 3.0 with H_3PO_4 . To 800 mL of this solution, add 200 mL of acetonitrile and mix. Store in a clean 1-L glass bottle. Filter the mobile phase and degas before using. Use within 30 days of preparation.

If necessary, connect the appropriate column to the injector and detector. Connect a 20- μL sample loop to the injector. Degas the mobile phase for approximately 5 min with helium or nitrogen immediately prior to use. Turn on the detector and the pump with the pump set for 1.5 mL/min flow. After approximately 15 min, measure the flow for 5 min with a 10-mL graduated cylinder. The flow rate should be 1.5 ± 0.1 mL/min. Adjust the flow rate if necessary to obtain a flow rate within these limits.

- c. HPLC Identity Confirmation: Prepare HPLC mobile phase for the initial identity confirmation using a Supelco LC-1 column by dissolving 6.0 g of sodium lauryl sulfate and 1.0 g of tetrabutylammonium nitrate in 1,000 mL of deionized water. Add 20 mL of glacial acetic acid to the solution and mix. Store in a clean glass bottle. Filter the mobile phase and degas before using. Use within 30 days of preparation.

If necessary, connect column to the injector and detector. Connect a 20- μL sample loop to the injector. Degas the mobile phase for approximately 5 min with helium or nitrogen immediately prior to use. Turn on the detector and the pump with the pump set for 1.0 mL/min flow. After approximately 15 min, measure the flow for 5 min with a 10-mL graduated cylinder. The flow rate should be 1.0 ± 0.1 mL/min. Adjust the flow rate if necessary to obtain a flow rate within these limits.

After the pump has been on for about 30 min, adjust the detector zero with the detector attenuation set at the appropriate attenuation. Adjust the recorder to electrical zero at "0" chart units. Adjust the detector zero to slightly above the electrical zero position with the recorder balance control.

4. Analysis of Samples: NMR is performed for structural confirmation. HPLC is performed to quantitatively determine the concentration of

pyridostigmine bromide and confirm the identity of the pyridostigmine bromide in the samples.

- a. NMR: When the response for the sample being analyzed has been maximized, proceed with the analysis. Multiple acquisitions (≥ 300 transients) are generally required. Spectra will be printed on standard NMR paper and computer referenced to the chemical shift of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) contained in the deuterium oxide. A listing of shifts and parameters used will be obtained.
- b. Quantitative HPLC: The following is a set of HPLC conditions that have been found to be satisfactory for quantitative analysis of pyridostigmine bromide by HPLC⁽¹⁾:

Column: Dupont Zorbax C8 or equivalent, 250-mm long x 4.6-mm inner diameter (I.D.) with 5 micron particle size.

Mobile Phase: 80 percent 0.05 M KH_2PO_4 with 3.0 mM tetramethylammonium chloride, pH 3.0, 20 percent acetonitrile (see Section D.3.b).

Detector: UV @ 269 nm.

Flow Rate: 1.5 mL/min.

Injection Volume: 20 μL .

For quantitative analysis of pyridostigmine bromide samples, transfer 1-mL duplicate aliquots of each pyridostigmine bromide standard to autosampler vials and place the vials in the autosampler in ascending concentration order. Set up the data system to acquire data for each standard as described in the data system instruction manual. Transfer 1-mL duplicate aliquots of each sample to autosampler vials and place the vials in the autosampler. For every ten samples to be analyzed, analyze one blank sample and one standard. Analyze under the same conditions used for the initial calibration standards.

- c. HPLC Identity Confirmation: For confirmation of the identity of pyridostigmine bromide by HPLC, a second set of HPLC conditions is employed. HPLC confirmation of the identity of pyridostigmine bromide is performed by analysis under a second set of HPLC conditions. Compare the retention times and relative responses of the pyridostigmine bromide reference standard and sample peak for structural confirmation in conjunction with the first set of HPLC results and NMR conclusions.

Column: Supelco LC-1, 250-mm long x 4.6-mm I.D. with 5 micron particle size.

Mobile Phase: 60 percent buffer (see Section D.3.c), 40 percent acetonitrile.

Detector: UV @ 254 nm.

Flow Rate: 1.0 mL/min.

Injection Volume: 20 μ L.

For confirmation purposes, analyze a pyridostigmine bromide standard and a sample from the formulation under these HPLC conditions.

5. Data Reduction: The NMR spectra obtained in Section D.4.a are compared with the reference NMR spectra for pyridostigmine bromide to verify structural identity. The HPLC samples analyzed in Section D.4 are compared with results obtained from known reference standards to determine concentration.

- a. NMR: Compare the NMR spectrum for the sample with the spectrum obtained for the pyridostigmine bromide reference standard. Verify correspondence of chemical shifts, multiplicities, and intensities for structural verification in conjunction with HPLC findings.
- b. Quantitative HPLC: Obtain printouts of the peak area ratios for each standard and sample as described in the instruction manual. Prepare a standard curve from the peak area ratios versus concentration of the standards.

Determine the pyridostigmine bromide concentration in the samples and control standards using the standard curve. If necessary, correct for any dilution made to the samples prior to analysis.

If the response for any of the control standards varies from the predicted response by more than ± 10 percent, then the samples associated with that standard are reanalyzed.

- c. HPLC Identity Confirmation: HPLC confirmation of the identity of pyridostigmine bromide is performed by analysis under a second set of HPLC conditions. Compare the retention times and relative responses of the pyridostigmine bromide reference standard and sample peak for structural confirmation in conjunction with the first set of HPLC results and NMR conclusions.

- d. HPLC Dose Verification: The identity of pyridostigmine bromide used during dose administration is verified by analyzing the administered dosage formulation by the HPLC method described in Section D.4.b. The response is compared to that obtained from a series of standards prepared from the analytical reference material to verify identity.

E. Reference:

"Assay of Syrup Preparation of Pyridostigmine Bromide, Syrup Mestinon, WR-250710AJ, BL08189," Draft Report No. 509, Contract No. DAMD17-85-C-5141, SRI International Project No. 8504, July 25, 1985.

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METHOD FOR ANALYZING DIAZEPAM
BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

- A. Statement of Work: This method describes the method for the quantitative analysis of diazepam in an injectable multi-solvent solution. The prepared sample is analyzed by high performance liquid chromatography (HPLC). The sample preparation and analysis methods detailed here were developed in support of on-going tasks at the MREF.
- B. Equipment: Freezer, refrigerator, labels, first aid kit, plastic-backed, absorbent paper, brown paper, squirt bottles, wiping tissues, beakers, bottles, maxi-vials, pipettes, pipette bulbs, tissue paper, laboratory coat, safety shoes, safety glasses, spatula, stainless-steel pans, glass stir rods, syringes, needles, forceps, scrub suit, and latex gloves.
- C. Area Set Up: An area in Room 46 or another approved room will be used to prepare calibration standards and perform spiking and extraction procedures.

The hood areas for solvent handling are covered with plastic-backed, absorbent paper. All materials for sample preparation are located in or near the hood area.

- D. Equipment Preparation:
1. Column Check: The integrity of the column needs to be checked before samples are analyzed. This is accomplished by analyzing a column test mix with appropriate conditions and comparing the resulting chromatogram with that of the sample chromatogram. The test mix and sample chromatogram are shipped with each column.
 2. Instrument Preparation: The HPLC is prepared for use with the following recommended initial settings:
 - a. Column - 8 cm x 4 mm inside diameter (I.D.) Zorbax ODS Cartridge Column with 5 μ m particle size.
 - b. Guard Column - 1.25 cm x 4 mm I.D. Zorbax ODS Cartridge Guard Column with 5 μ m particle size.
 - c. Mobile Phase: 50 percent buffer/50 percent acetonitrile.
 - d. Mobile Phase Flow Rate: 1.0 mL/min.
 - e. Injection Loop: 20 μ L volume.
 - f. Detector Wavelength: 300 nm.
 - g. Absorbance Units Full Scale (A.U.F.S.) - 0.02.

3. Column Conditioning: The column needs approximately 30 min of conditioning before it can be used to analyze samples. This conditioning insures that all stationary phase has been "washed" with the mobile phase producing a homogeneous environment.

E. Solution Preparation:

1. Mobile Phase Buffer: Accurately weigh 2.44 ± 0.01 g potassium phosphate dibasic and 15.42 ± 0.01 g ammonium acetate onto weighing paper. Quantitatively transfer these chemicals into a 2-L volumetric flask containing approximately 500-mL millipore water. Dilute to volume with millipore water. Mix well and pH solution to pH 6.8 with a 0.1 M-phosphoric acid solution. Filter the resulting solution through a $0.45 \mu\text{m}$ filter.

Prepare a solution which is approximately 0.1 M H_3PO_4 by dispensing approximately 0.5 mL of H_3PO_4 into a 50-mL beaker containing 10-mL millipore water. Mix well. CAUTION: Process is exothermic.

2. Multisol solvent: The multisol solvent is prepared by dispensing 200-mL propylene glycol, 50-mL denatured alcohol, and 7.5-mL benzyl alcohol into a 500-mL volumetric flask and diluting to volume with millipore water and vortexing to insure complete mixing.
3. Diazepam Stock Solution: The diazepam stock solution is prepared from pure crystalline diazepam supplied by Hoffman-La Roche. Standards should be prepared in a range of concentrations which bracket the nominal concentrations of the samples. An example of a suitable dilution scheme follows.
 - a. 1.0-mg/mL Diazepam Stock Solution: Accurately weigh 10 ± 0.1 mg of diazepam onto weighing paper. Quantitatively transfer the diazepam into a 10-mL volumetric flask containing approximately 5-mL Multisol. Mix well using a vortex mixer. Multisol is a viscous liquid and requires a lot of mixing to get diazepam into solution until dissolved. Dilute to volume with Multisol® and mix again. Specific density is 1.007 at 25 degrees C.
 - b. Preparation of Diazepam Analytical Standards:
 - (1) 0.700-mg/mL Analytical Standard: Aliquot 0.70-mL of the diazepam stock solution into each of two 1.8 mL auto-injection vials and dilute with 0.30-mL of mobile phase. Label the vials with the following information:
(1) contents, (2) concentration of analyte (3) date of formulation. Store in the freezer at -20°C until use.
 - (2) 0.600-mg/mL Analytical Standard: Aliquot 0.60-mL of the diazepam stock solution into each of two 1.8 mL auto-injection vials and dilute with 0.40-mL of mobile phase. Label the vials with the following information:

- (1) contents, (2) concentration of analyte (3) date of formulation. Store in the freezer at -20 C until use.
- (3) 0.500-mg/mL Analytical Standard: Aliquot 0.50-mL of the diazepam stock solution into each of two 1.8 mL auto-injection vials and dilute with 0.50-mL of mobile phase. Label the vials with the following information: (1) contents, (2) concentration of analyte, (3) date of formulation. Store in the freezer at -20 C until use.
- (4) 0.400-mg/mL Analytical Standard: Aliquot 0.40-mL of the diazepam stock solution into each of two 1.8 mL auto-injection vials and dilute with 0.60-mL of mobile phase. Label the vials with the following information: (1) contents, (2) concentration of analyte, (3) date of formulation. Store in the freezer at -20 C until use.
- (5) 0.300-mg/mL Analytical Standard: Aliquot 0.30-mL of the diazepam stock solution into each of two 1.8 mL auto-injection vials and dilute with 0.70-mL of mobile phase. Label the vials with the following information: (1) contents, (2) concentration of analyte, (3) date of formulation. Store in the freezer at -20 C until use.
- (6) 0.000-mg/mL Analytical Standard: Aliquot 0.10-mL of the multisol stock solution into each of two 1.8 mL auto-injection vials and dilute with 0.90-mL of mobile phase. Label the vials with the following information: (1) contents, (2) concentration of analyte (3) date of formulation. Store in the freezer at -20 C until use.
4. Collection and Storage of Samples: Samples are collected in 2-mL GC vials treated with hexamethyldisiloxane (HMDS) to prevent reaction with active sites in the glass. Diazepam samples generated this way can be stored in the Revco freezer at -70 C for up to 60 days until analyzed.
5. Sample Preparation: The samples are diluted to a concentration within the calibration range of the instrument before analysis. The same dilution procedures are used to dilute the samples as were used to prepare the calibration standards. Aliquot 0.10-mL of the diazepam sample into each of two 1.8 mL auto-injection vials and dilute with 0.90-mL of mobile phase. Label the vials with the following information: (1) contents, (2) concentration of analyte, (3) date of formulation. Store in the freezer at -20 C until use.
6. Calibration: Instrument calibration is performed when quantitation of samples is required by injecting 20 μ L each of analytical standard prepared in Section G.4 using an autosampler. A complete set of calibration standards is analyzed prior to analysis of any sample. Once the calibration of the instrument has been checked, the samples

are analyzed with at least every sixth sample being a calibration standard to check the calibration of the instrument. A complete set of calibration standards is analyzed following the last sample. All calibration standards analyzed are used to develop a complete calibration curve for quantitation of the samples. No sample amount may be reported that exceeds the range of the calibration standards. Samples that yield responses less than the calibration range will be reported as less than the lower quantitation limit. Any sample response that exceeds the largest calibration standard will be reported as greater than the highest calibration standard, and must be either diluted to within range or the calibration range extended for quantification of the sample.

7. Analysis of Samples: Samples and calibration standards are analyzed using the sample procedures. At least every sixth analysis should be a standard.

8. Calculations:

- a. The samples are analyzed using a regression analysis with internal standards.
- b. Using a linear regression program, generate the slope, intercept, and correlation coefficient for diazepam in the calibration data.
- c. Enter the peak area of diazepam as the ordinate (x-value) and the corresponding standard concentration as the abscissa (y-value).
- d. Enter each data point obtained from the calibration standards and calculate percent relative standard deviation (% RSD) between replicate standards. Do not include the blank in the calibration calculations as this will weigh the regression toward zero.
- e. If a regression program is not available, program the following calculations:

$$b = \frac{[(\Sigma y)(\Sigma x^2) - (\Sigma x)(\Sigma xy)]}{[n(\Sigma x^2) - (\Sigma x)^2]}$$

$$a = \frac{[n(\Sigma xy) - (\Sigma x)(\Sigma y)]}{[n(\Sigma x^2) - (\Sigma x)^2]}$$

$$r = \frac{[n(\Sigma xy) - (\Sigma x)(\Sigma y)]}{[(n(\Sigma x^2) - (\Sigma x)^2)^{1/2}(n(\Sigma y^2) - (\Sigma y)^2)^{1/2}]}$$

where,

$y = ax + b$
a = slope
b = y-intercept
r = correlation coefficient
x = peak area (diazepam)
y = concentration of agent in mg/mL
n = number of replicates

- f. Identify the analyte peak in the sample chromatograms; record the peak area. Using the regression values calculated from the calibration data, calculate the found concentration for each sample using the formula above.
9. Column Clean-up: After each analysis day, the column needs to be flushed with a mixture of acetonitrile, methanol and water. Flush the column with 33:33:34 mixture of ACN/MeOH/H₂O for approximately 30 min with a flow rate of 2-mL per min.
10. Instrument Shut-Down: When the instrument is not to be used for extended periods of time, the system must be shut down following manufacturer's instructions to ensure column life and instrument stability. The column clean-up procedure is followed, and the column is stored with 100 percent ACN wetting the stationary phase.

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APPENDIX C

**GROSS PATHOLOGY OBSERVED AT THE TIME
OF NECROPSY OF EACH ANIMAL**

INDIVIDUAL ANIMAL GROSS PATHOLOGY
ALPHANUMERIC ORDER BY TATTOO

<u>Tattoo #</u>	<u>Gross Pathology/Comment</u>
G244	No significant lesions.
G654	Lungs - adhesions to thoracic wall.
G708	Lungs - Adhesions to thoracic wall; Heart, right ventricle, epicardium - hemorrhage, focal, 1-2 mm
G757	No significant lesions.
G867	No significant lesions.
G923	Skeletal muscle - necrosis, injection sites. Note: minor facial bruises (not saved).
H074	No significant lesions. Note: all tissues autolyzed.
H167	No significant lesions. Note: minor facial bruises (not saved).
H227	Lungs - adhesions to thoracic wall.
H237	No significant lesions.
H258	No significant lesions.
H263	No significant lesions.
H264	No significant lesions.
H282	No significant lesions.
H300	No significant lesions.
H309	No significant lesions.
H355	No significant lesions.
H398	Ileum - discoloration, dark red. Note: Left eye ruptured during necropsy.
H413	No significant lesions. Note: Superficial contusion, left chin (not saved).
H432	No significant lesions.
H436	Urinary Bladder - thick; blood in lumen.
H444	No significant lesions.
H453	Lungs, mottled, dark red.
H472	Lungs - adhesions to thoracic wall.
H482	No significant lesions.
H483	Kidney, left - enlarged, dark; Adrenal, left - enlarged.
H486	No significant lesions.
H489	No significant lesions.
H525	No significant lesions.
H585	No significant lesions.
H602	Heart - pericardial adhesions
H612	No significant lesions.
H632	Skeletal muscle - necrotic tract, injection sites; Skeletal muscle, temporal - bilateral necrosis.
H789	No significant lesions.

<u>Tattoo #</u>	<u>Gross Pathology/Comment</u>
H816	Lungs - adhesions to thoracic wall.
H818	Skeletal muscle - necrosis, injection sites. Note: facial bruising; red fluid in stomach.
H831	No significant lesions.
H843	No significant lesions.
I438	No significant lesions.
5R2	Skeletal muscle - necrosis, injection sites.
5U3	No significant lesions.
5WT	No significant lesions.
6NL	Lungs - cysts, multiple, approximately 4 mm diameter.
6PJ	No significant lesions.
6RA	No significant lesions.
6RB	No significant lesions.
6S4	No significant lesions.
6T4	No significant lesions.
6TM	No significant lesions.
6TR	No significant lesions.
6TY	No significant lesions.
6VZ	No significant lesions.
6WB	Stomach - discoloration, focal, red.
6WG	Skeletal muscle - hemorrhage, injection sites.
6W2	Skeletal muscle - necrosis, injection site.
6W6	No significant lesions.
6W8	No significant lesions.
6XC	No significant lesions. Note: distal jejunum, cecum, and colon filled with tarry digesta.
6XM	Skeletal muscle - necrosis, injection sites.
6XR	Thyroid - enlarged, slight; Mesenteric lymph node -enlarged.
6Z1	Lungs - adhesions to diaphragm.
66P	No significant lesions.
7AC	No significant lesions.
7AH	Ileum - hemorrhage, focal.
7AL	Lungs - mottled, dark red.
7AU	No significant lesions.
7BK	No significant lesions. Note: superficial facial bruises (not saved).
7BM	No significant lesions.
7BY	No significant lesions.
7CA	Adrenal, left - enlarged; Lungs - discoloration, red; Stomach - discoloration, red.
7CC	Lungs - mottled, dark red.
7CG	No significant lesions.
7CK	No significant lesions.

<u>Tattoo #</u>	<u>Gross Pathology/Comment</u>
7CU	No significant lesions.
7C4	Lungs - mottled, dark red.
7C6	Ileum - hemorrhage, focal.
7C9	Heart, epidardium - hemorrhage, multifocal. Note: blood on face; hemorrhage in fascia over biceps femoris.
7D4	No significant lesions.
7D6	Skin - ulcer, right hip.
71D	No significant lesions.
71G	No significant lesions.
73C	No significant lesions.
73P	No significant lesions.
74A	No significant lesions.
74H	No significant lesions.
75F	No significant lesions.
75G	No significant lesions.
75H	No significant lesions.
75P	No significant lesions.
75U	Heart - hemorrhage, multifocal.
75Z	No significant lesions.
76P	No significant lesions.
77K	No significant lesions.
77L	No significant lesions. Note: superficial bruises (face and knee) not saved.
77V	No significant lesions.
78J	No significant lesions.
78S	Ileum - discoloration, black.
78V	No significant lesions.
78X	No significant lesions.
78Y	No significant lesions.
79C	No significant lesions.
79P	No significant lesions.
79Y	No significant lesions.

APPENDIX D

DATA AND STATISTICAL ANALYSES

TABLE D-1. PHASE I DATA LISTING

Treatment Group	Animal	Date	Target GD Dose ($\mu\text{g/kg}$)	Calculated* GD Dose ($\mu\text{g/kg}$)	48-Hour Results	Body Weight (kg)	Baseline AChE Activity (U/mL)
Atropine/2-PAM	H489	2/23/93	9.2	9.1	Alive	7.9	6.62
Atropine/2-PAM	H486	2/23/93	15.0	14.9	Alive	7.2	10.21
Atropine/2-PAM	G708	2/25/93	16.0	15.6	Dead	9.0	8.98
Atropine/2-PAM	7D4	3/02/93	17.0	17.0	Alive	8.2	9.78
Atropine/2-PAM	79C	3/09/93	18.0	18.1	Alive	7.7	11.72
Atropine/2-PAM	7BY	3/09/93	20.0	20.3	Alive	8.0	10.28
Atropine/2-PAM	6NL	2/25/93	21.0	21.7	Alive	8.2	10.11
Atropine/2-PAM	G757	3/04/93	23.0	23.1	Dead	8.2	9.98
Atropine/2-PAM	H263	3/02/93	25.0	24.9	Dead	8.0	9.22
Atropine/2-PAM	7BM	3/04/93	27.0	26.8	Dead	8.1	8.43
Untreated	G867	2/25/93	4.5	3.4	Alive	9.2	11.22
Untreated	6TM	2/23/93	5.5	5.2	Dead	8.4	10.68
Untreated	H444	3/02/93	5.3	5.4	Alive	8.2	8.28
Untreated	H413	2/25/93	5.8	5.7	Alive	8.7	9.72
Untreated	77L	2/23/93	8.3	6.9	Dead	6.9	9.10
Untreated	7CG	3/02/93	7.9	7.8	Dead	7.1	10.97

* Calculated doses based on weight losses of syringes and chemical analysis of dosing solution.

TABLE D-2. COMPARISON OF RESULTS OF PROBIT ANALYSES OF DATA BASED ON SEPARATE MODELS FITTED TO EACH UNTREATED GROUP

Treatment Group	N	Slope	Slope 95% Confidence Limits	LD ₅₀ (µg/kg)	LD ₅₀ 95% Confidence Limits
89-09, 89-12 Untreated	19	14.0	2.5, 25.4	6.7	5.8, 9.2
92-30 Untreated	6	13.4	-8.9, 35.7	5.7	NC

NC - The confidence interval could not be calculated because the slope was not significantly different from zero.

TABLE D-3. SUMMARY OF PROBIT ANALYSES OF TWO UNTREATED AND TWO ATROPINE/2-PAM TREATED GROUPS BASED ON SEPARATE MODELS FITTED TO EACH GROUP

Treatment Group	N	Slope	Slope 95% Confidence Limits	LD ₅₀ (µg/kg)	LD ₅₀ 95% Confidence Limits	Protective Ratio (95% Confidence Limits)
85-18 Untreated	36	10.3	2.9, 17.7	15.1	12.5, 17.5	
85-18, ATR/2-PAM	28	7.2	-1.4, 15.9	25.2	NC	1.7 (1.4,2.0)
89-08, 89-12, 92-30 Untreated	25	12.1	3.2, 20.9	6.5	5.6, 8.0	
92-30, ATR/2-PAM	10	8.1	-2.7, 18.9	20.7	NC	3.2 (2.4,4.2)

NC - The confidence interval could not be calculated because the slope was not significantly different from zero.

TABLE D-4. PERCENT AChE INHIBITION IN RESPONSE TO i.m. PYR
IN PHASE II EXPERIMENTS

Date	Animal	Target Pyridostigmine Dose ($\mu\text{g/kg}$)	Time (min)	Percent AChE Inhibition
03/24/93	73C	8.4	5	0.5
			10	-4.4
			20	NS ^(a)
			30	1.9
			45	10.2
			60	6.0
			90	-4.1
03/24/93	H398	8.4	5	-0.6
			10	1.8
			20	9.0
			30	8.7
			45	8.0
			60	12.0
			90	6.4
03/29/93	5R2	8.4	5	0.4
			10	4.7
			20	0.0
			30	5.7
			45	6.6
			60	7.3
			90	9.1
03/29/93	6RB	8.4	5	9.9
			10	9.6
			20	10.7
			30	13.3
			45	14.9
			60	15.1
			90	10.0
03/29/93	74A	8.4	5	5.0
			10	5.6
			20	8.5
			30	5.7
			45	4.5
			60	15.6
			90	7.4

TABLE D-4.
(Continued)

Date	Animal	Target Pyridostigmine Dose ($\mu\text{g/kg}$)	Time (min)	Percent AChE Inhibition
03/29/93	H237	8.4	5	5.3
			10	10.7
			20	9.8
			30	12.7
			45	8.8
			60	6.1
			90	5.3
03/31/93	6TR	8.4	5	-0.8
			10	2.2
			20	4.1
			30	7.4
			45	6.5
			60	3.8
			90	2.4
03/31/93	6WG	8.4	5	2.5
			10	3.0
			20	5.0
			30	6.0
			45	3.3
			60	10.3
			90	8.9
03/31/93	H843	8.4	5	1.9
			10	4.9
			20	6.5
			30	7.3
			45	6.9
			60	4.9
			90	1.3
03/31/93	I438	8.4	5	5.8
			10	5.4
			20	5.8
			30	8.1
			45	8.9
			60	11.8
			90	7.5

TABLE D-4.
(Continued)

Date	Animal	Target Pyridostigmine Dose ($\mu\text{g/kg}$)	Time (min)	Percent AChE Inhibition
03/02/93	5R2	10.5	5	6.1
			10	9.9
			20	12.9
			30	15.5
			45	7.8 ^(b)
			60	15.7
			90	12.6
03/02/93	6RB	10.5	5	1.4
			10	4.9
			20	6.1
			30	8.8
			45	11.7
			60	12.9
			90	10.1
03/04/93	6WG	18.0	5	5.7
			10	14.8
			20	15.0
			30	12.9
			45	14.5
			60	14.9
			90	11.0
03/04/93	74A	18.0	5	6.7
			10	10.6
			20	13.4
			30	15.1
			45	13.5
			60	8.0 ^(b)
			90	13.6
03/15/93	73C	25.0	5	-0.1
			10	7.9
			20	13.7
			30	19.0
			45	21.0
			60	16.9
			90	15.9

TABLE D-4.
(Continued)

Date	Animal	Target Pyridostigmine Dose ($\mu\text{g}/\text{kg}$)	Time (min)	Percent AChE Inhibition
03/15/93	H398	25.0	5	0.8
			10	11.5
			20	20.9
			30	21.1
			45	21.2
			60	17.7
			90	13.5
03/22/93	6TR	26.0	5	6.4
			10	16.4
			20	22.7
			30	27.7
			45	22.2
			60	15.6
			90	11.3
03/22/93	6WG	26.0	5	10.7
			10	15.4
			20	19.7
			30	24.2
			45	23.1
			60	20.4
			90	14.0
03/22/93	H843	26.0	5	16.2
			10	22.8
			20	26.4
			30	26.2
			45	26.5
			60	24.4
			90	17.4
03/22/93	I438	26.0	5	9.2
			10	15.6
			20	20.9
			30	21.7
			45	19.4
			60	18.7
			90	15.3

TABLE D-4.
(Continued)

Date	Animal	Target Pyridostigmine Dose ($\mu\text{g/kg}$)	Time (min)	Percent AChE Inhibition
03/17/93	5R2	27.0	5	13.6
			10	20.3
			20	30.2
			30	26.7
			45	30.5
			60	30.5
			90	24.3
03/17/93	6RB	27.0	5	17.0
			10	23.1
			20	25.2
			30	24.5
			45	30.2
			60	24.2
			90	18.4
03/17/93	74A	27.0	5	14.5
			10	22.1
			20	22.6
			30	25.3
			45	28.8
			60	23.8
			90	19.2
03/17/93	H237	27.0	5	13.7
			10	19.2
			20	25.9
			30	25.1
			45	24.3
			60	22.7
			90	18.3
03/10/93	6TR	33.0	5	9.8
			10	17.4
			20	20.4
			30	23.7
			45	25.3
			60	20.9
			90	14.5

TABLE D-4.
(Continued)

Date	Animal	Target Pyridostigmine Dose ($\mu\text{g/kg}$)	Time (min)	Percent AChE Inhibition
03/10/93	H843	33.0	5	19.4
			10	25.3
			20	33.7
			30	35.0
			45	33.1
			60	29.4
			90	25.7
03/08/93	H237	80.0	5	17.1
			10	31.7
			20	39.9
			30	46.3
			45	42.5
			60	40.0
			90	31.2
03/08/93	I438	80.0	5	15.9
			10	34.9
			20	45.6
			30	46.6
			45	42.0
			60	36.0
			90	28.0

(a) No blood sample obtained due to problems with catheter patency.

(b) Points flagged as outliers; not used in statistical analyses.

TABLE D-5. EMPIRICALLY OBSERVED AND QUADRATICALLY SMOOTHED
VALUES OF CMAX AND TMAX FOR PHASE II EXPERIMENTS

Date	Animal	Target PYR Dose ($\mu\text{g/kg}$)	Empirical Cmax	Smoothed Cmax	Empirical tmax	Smoothed tmax
03/24/93	73C	8.4	10.2	6.8	45	47.1
03/24/93	H398	8.4	12.0	11.2	60	55.7
03/29/93	5R2	8.4	9.1	9.2	90*	120.4
03/29/93	6RB	8.4	15.1	14.7	60	50.7
03/29/93	74A	8.4	15.6	9.7	60	64.6
03/29/93	H237	8.4	12.7	9.8	30	34.5
03/31/93	6TR	8.4	7.4	6.4	30	49.7
03/31/93	6WG	8.4	10.3	9.3	60	129.4
03/31/93	H843	8.4	7.3	7.0	30	42.2
03/31/93	I438	8.4	11.8	9.9	60	58.6
03/02/93	5R2	10.5	15.7	17.1	60	54.7
03/02/93	6RB	10.5	12.9	12.6	60	62.7
03/04/93	6WG	18.0	15.0	15.4	20	49.6
03/04/93	74A	18.0	15.1	16.0	30	58.3
03/15/93	73C	25.0	21.0	21.4	45	57.6
03/15/93	H398	25.0	21.2	22.7	45	51.7
03/22/93	6TR	26.0	27.7	23.6	30	45.0
03/22/93	6WG	26.0	24.2	23.6	30	48.1
03/22/93	H843	26.0	26.5	27.1	45	44.7
03/22/93	I438	26.0	21.7	21.4	30	49.6
03/17/93	5R2	27.0	30.5	31.9	45	53.9
03/17/93	6RB	27.0	30.2	27.5	45	50.4
03/17/93	74A	27.0	28.8	27.1	45	49.1
03/17/93	H237	27.0	25.9	25.6	20*	48.1
03/10/93	6TR	33.0	25.3	24.7	45	48.3
03/10/93	H843	33.0	35.0	34.3	30	48.7
03/08/93	H237	80.0	46.3	45.7	30	50.4
03/08/93	I438	80.0	46.6	45.1	30	46.9

* Results flagged as outliers based on empirical tmax values.

TABLE D-6. DATA LISTING OF PHASE III RESULTS

Animal	Body Weight (kg)	Date	GD* Dose ($\mu\text{g/kg}$)	PYR Dose ($\mu\text{g/kg}$)	Baseline AChE Activity (U/mL)	Percent AChE Inhibition	48-Hour Results
75G	6.6	04/20/93	31.9	0.0	10.2	3.7	Died
78J	7.6	04/20/93	32.1	0.0	10.7	2.8	Died
7CA	7.3	05/25/93	30.9	0.0	9.4	2.7	Died
H355	7.9	05/25/93	32.2	0.0	8.7	2.8	Died
6RA	7.2	04/20/93	31.6	4.0	13.2	10.7	Lived
79Y	7.4	04/20/93	32.0	4.0	9.4	6.4	Lived
G654	8.4	04/27/93	32.6	4.0	9.5	5.5	Lived
H831	7.9	04/27/93	32.5	4.0	9.2	6.9	Died
78X	7.6	05/04/93	32.2	4.0	11.7	5.2	Lived
H818	7.4	05/04/93	31.4	4.0	8.0	7.9	Lived
6WB	8.1	05/18/93	33.3	4.0	10.2	3.1	Lived
75F	7.8	05/18/93	32.5	4.0	9.7	5.6	Lived
76P	7.3	05/25/93	32.1	4.0	10.4	9.5	Died
7AH	7.3	06/08/93	32.6	4.0	9.9	8.4	Lived
77K	7.8	04/06/93	31.4	8.4	9.0	13.9	Lived
G244	7.4	04/06/93	32.2	8.4	11.5	6.0	Lived
6Z1	7.9	04/13/93	32.5	8.4	9.5	12.9	Lived
78Y	8.0	04/13/93	32.5	8.4	10.0	14.9	Lived
6PJ	7.3	04/27/93	32.2	8.4	10.1	18.6	Lived
H436	7.7	04/27/93	30.6	8.4	11.8	9.1	Lived
6W2	7.6	05/18/93	32.0	8.4	11.9	13.0	Lived
H602	7.9	05/18/93	32.9	8.4	8.0	10.7	Lived
H432	7.8	05/25/93	31.9	8.4	9.3	12.4	Died
6XR	7.4	06/08/93	31.3	8.4	8.8	9.7	Lived
77V	6.3	04/06/93	32.9	24.0	11.6	28.8	Died
H632	7.5	04/06/93	32.6	24.0	10.8	32.7	Lived
6XC	7.4	04/13/93	33.0	24.0	8.6	23.8	Died
H227	7.4	04/13/93	33.0	24.0	8.5	23.2	Lived
71D	7.6	05/04/93	32.0	24.0	8.9	28.9	Lived
H789	6.9	05/04/93	32.0	24.0	8.4	29.8	Lived
78S	7.4	05/18/93	33.3	24.0	11.3	29.1	Lived
H282	8.1	05/18/93	32.6	24.0	8.9	33.4	Lived
H816	7.2	05/25/93	32.7	24.0	7.4	30.1	Died
H483	7.5	06/08/93	31.8	24.0	9.0	27.6	Lived

* Targeted GD dose was 32.5 $\mu\text{g/kg}$. GD doses in this column are based on weight losses of syringes and initial chemical concentration analysis of the dosing solution.

TABLE D-7. PERCENT AChE INHIBITION IN RESPONSE TO i.g. PYR
IN PHASE IV EXPERIMENTS

PYR Dose ($\mu\text{g/kg}$)	Date	Animal	Time (min)	Percent AChE Inhibition
0	07/16/93	6RB	30	-1.4
			45	-2.7
			60	0.7
			75	0.6
			90	0.2
			105	-3.7
			120	1.5
			135	-1.3
			150	-0.1
			165	-3.4
			180	NS ^(a)
0	07/16/93	73C	30	-7.6
			45	-7.5
			60	-6.5
			75	-6.4 ^(b)
			90	-16.1 ^(b)
			105	NS
			120	NS
			135	NS
			150	NS
			165	NS
			180	NS
50	06/29/93	5R2	30	32.0
			45	27.3
			60	28.6
			75	24.5
			90	22.1
			105	27.1
			120	21.5
			135	19.4
			150	19.5
			165	19.2
			180	13.6

^(a) No sample obtained.

^(b) Hemolyzed sample.

TABLE D-7.
(Continued)

PYR Dose ($\mu\text{g/kg}$)	Date	Animal	Time (min)	Percent AChE Inhibition
50	06/29/93	6WG	30	-6.8
			45	-4.1
			60	-4.2
			75	2.2
			90	0.9
			105	5.9
			120	4.8
			135	4.5
			150	5.6
			165	5.5
			180	4.3
50	07/08/93	5R2	30	-0.7
			45	-4.5
			60	3.7
			75	1.1
			90	2.0
			105	4.8
			120	6.1
			135	9.7
			150	12.8
			165	3.7
			180	6.1
50	07/08/93	6WG	30	0.4
			45	3.6
			60	0.3
			75	3.3
			90	-0.3
			105	1.2
			120	9.8
			135	11.0
			150	8.7
			165	11.7
			180	15.3
			195	10.7
			210	9.2

TABLE D-7.
(Continued)

PYR Dose ($\mu\text{g/kg}$)	Date	Animal	Time (min)	Percent AChE Inhibition
50	07/16/93	74A	30	4.8
			45	3.6
			60	10.8
			75	7.2
			90	9.1
			105	14.1
			120	11.7
			135	11.8
			150	10.0
			165	9.7
			180	10.2
50	07/16/93	H398	30	3.3
			45	5.5
			60	3.4
			75	0.9
			90	2.2
			105	5.0
			120	9.1
			135	14.4
			150	19.3
			165	19.0
			180	19.6
			195	17.7
50	07/27/93	74A	30	4.1
			45	6.3
			60	8.6
			75	7.2
			90	5.5
			105	8.6
			120	11.5
			135	7.6
			150	11.6
			165	9.4
			180	10.9
			195	6.5

TABLE D-7.
(Continued)

PYR Dose ($\mu\text{g/kg}$)	Date	Animal	Time (min)	Percent AChE Inhibition
50	07/27/93	H398	30	2.8
			45	-3.4
			60	2.3
			75	5.8
			90	8.9
			105	15.2
			120	11.7
			135	10.6
			150	11.5
			165	14.3
			180	14.7
			195	12.0
125	06/22/93	5R2	30	-1.4
			45	0.1
			60	10.6
			75	11.6
			90	12.6
			105	17.5
			120	26.4
			135	22.5
			150	25.2
			165	25.4
			180	24.8
125	06/22/93	6WG	30	27.1
			45	27.7
			60	28.4
			75	26.9
			90	28.5
			105	28.6
			120	23.3
			135	21.8
			150	19.8
			165	19.6
			180	23.9
			195	11.3
			210	12.5

TABLE D-8. EMPIRICALLY OBSERVED AND QUADRATICALLY SMOOTHED VALUES OF C_{max} AND t_{max} FOR EACH ANIMAL AND PYR DOSE FOR PHASE IV EXPERIMENTS

Date	Animal	PYR Dose (μ g/kg)	Empirical C _{max}	Smoothed C _{max}	Empirical t _{max}	Smoothed t _{max}
06/22/93	5R2	125	26.4	25.6	120	174.5
06/22/93	6WG	125	28.6	27.9	105	54.0
06/29/93	5R2 ^(a)	50	32.0	30.3	30	30.0
06/29/93	6WG	50	5.9	5.5	105	147.6
07/08/93	5R2	50	12.8	7.4	150	158.6
07/08/93	6WG	50	15.3	18.0	180	421.4
07/08/93	6WG ^(b)	50	15.3	11.4	180	190.0
07/16/93	6RB	0	1.5	-0.1	120	92.4
07/16/93	73C	0	-6.4	-5.5	75	51.9
07/16/93	74A	50	14.1	11.6	105	127.5
07/16/93	H398 ^(a)	50	19.6	22.3	180	195.0
07/27/93	74A	50	11.6	9.6	150	142.0
07/27/93	H398	50	15.2	13.5	105	170.2

^(a) AChE-I time course data were atypical and not downwardly concave within the time frame evaluated.

^(b) Regression rerun for animal 6WG omitting outlying data points at 90 and 105 min. The results of this rerun regression were used in the data analysis for this animal.

TABLE D-9. AChE INHIBITION AND LETHALITY RESULTS FOR PHASE IV EXPERIMENTS:
40 µg/kg PYR i.g.; 5 X 48-hr GD LD50 150 MIN FOLLOWING PYR;
ATR/2-PAM TREATMENT

Date	Animal	Body Weight (kg)	Baseline AChE Activity ^(a)			AChE-I ^(b)		Calculated ^(c) PYR Dose (µg/kg)	Calculated ^(c) GD Dose (µg/kg)	48-Hour Results
			-10 min (U/mL)	-5 min U/mL		-15 min (%)	-5 min (%)			
8/03/93	75H	6.1	10.1	9.8		-1.6	2.1	40	32.9	Alive
8/03/93	H167	6.4	7.9	7.7		0.3	-2.0	40	33.2	Alive
8/09/93	6TR	7.3	10.0	10.0		2.8	-1.8	40	32.2	Alive
8/09/93	5U3	7.3	10.0	10.1		9.4	12.4	40	32.1	Dead
8/10/93	H843	7.3	10.1	9.9		12.5	9.4	40	31.9	Alive
8/10/93	G923	7.3	11.3	11.2		4.2	3.1	40	31.8	Alive
8/23/93	H237	6.9	11.4	11.2		2.0	2.2	40	31.5	Alive
8/23/93	7CU	7.2	8.9	9.0		-3.0	-2.3	39	31.2	Alive
8/24/93	7BK	7.3	9.9	10.3		15.7	14.2	39	32.1	Dead
8/24/93	6XM	7.2	13.1	13.1		-5.6	-2.3	40	32.3	Alive

^(a)AChE activity measured in blood samples drawn approximately 10 and 5 min prior to the intragastric dosing of PYR.

^(b)AChE-I values of blood taken approximately 15 and 5 min prior to injection of GD (approximately 135 and 145 min after i.g. dosing of PYR). AChE-I calculations based on -5 min baseline AChE activity value.

^(c)Doses calculated from weight losses of syringes and concentrations of dosing solutions based on chemical analyses.

TABLE D-10. PHASE V DATA LISTING

Treatment Group	Date	Animal	Target		Body Weight (kg)	Percent AChE Inhibition	48-Hour Results	10-Day Results
			GD Dose ($\mu\text{g/kg}$)	Calculated* GD Dose ($\mu\text{g/kg}$)				
ATR/2-PAM/DZM	10/19/93	7CK	7.3	5.8	6.6	-2.3	Alive	Alive
ATR/2-PAM/DZM	10/12/93	71G	7.5	6.6	7.9	-1.3	Alive	Alive
ATR/2-PAM/DZM	09/21/93	78V	10.0	7.3	5.1	-0.2	Alive	Alive
ATR/2-PAM/DZM	09/28/93	6S4	10.5	10.9	8.2	3.2	Died	Died
ATR/2-PAM/DZM	09/24/93	75U	11.0	11.6	8.3	-0.1	Died	Died
ATR/2-PAM/DZM	09/14/93	7AU	12.0	12.3	7.7	1.0	Died	Died
ATR/2-PAM/DZM	09/10/93	6W6	15.0	15.3	6.9	1.6	Died	Died
ATR/2-PAM/DZM	09/03/93	7CC	18.0	17.9	6.5	-1.3	Alive	Died
ATR/2-PAM/DZM	09/07/93	H453	20.5	19.4	7.1	-2.2	Died	Died
ATR/2-PAM/DZM	08/31/93	H264	25.0	28.5	7.4	4.9	Died	Died
ATR/2-PAM	10/12/93	H482	5.5	5.7	8.4	-6.6	Alive	Alive
ATR/2-PAM	10/12/93	66P	7.0	6.9	7.3	0.4	Alive	Alive
ATR/2-PAM	10/19/93	75P	8.5	8.5	8.0	-2.3	Alive	Alive
ATR/2-PAM	09/28/93	7AC	8.0	7.9	8.0	0.8	Died	Died
ATR/2-PAM	09/24/93	74A	8.0	8.5	6.9	-2.8	Alive	Alive
ATR/2-PAM	09/21/93	6VZ	10.0	10.0	6.9	1.3	Died	Died
ATR/2-PAM	10/19/93	H258	13.0	13.2	7.0	-1.1	Died	Died
ATR/2-PAM	09/28/93	6RB	14.0	13.3	5.7	3.0	Died	Died

TABLE D-10.
(Continued)

Treatment Group	Date	Animal	Target GD Dose (μ g/kg)	Calculated* GD Dose (μ g/kg)	Body Weight (kg)	Percent AChE Inhibition	48-Hour Results	10-Day Results
PYR/ATR/2-PAM	08/31/93	H398	80.0	79.4	5.8	2.2	Alive	Died
PYR/ATR/2-PAM	09/14/93	6WG	130.0	129.4	6.0	9.8	Alive	Alive
PYR/ATR/2-PAM	09/03/93	6TY	160.0	158.3	6.9	7.8	Alive	Died
PYR/ATR/2-PAM	09/21/93	73C	175.0	173.8	7.0	7.0	Alive	Died
PYR/ATR/2-PAM	10/12/93	75Z	210.0	109.4	8.0	8.6	Alive	Alive
PYR/ATR/2-PAM	10/19/93	H525	215.0	212.3	7.4	9.5	Died	Died
PYR/ATR/2-PAM	09/10/93	6T4	200.0	199.7	7.5	7.9	Died	Died
PYR/ATR/2-PAM	09/28/93	73P	210.0	208.3	6.6	9.5	Died	Died
PYR/ATR/2-PAM	09/24/93	H074	210.0	208.4	8.0	7.5	Died	Died
PYR/ATR/2-PAM	09/07/93	H585	260.0	257.8	7.0	3.3	Died	Died
PYR/ATR/2-PAM/DZM	10/19/93	79P	60.0	60.3	7.8	5.4	Alive	Alive
PYR/ATR/2-PAM/DZM	10/12/93	H472	75.0	76.1	7.7	1.4	Died	Died
PYR/ATR/2-PAM/DZM	09/28/93	7C6	75.0	75.4	7.3	1.3	Alive	Died
PYR/ATR/2-PAM/DZM	08/31/93	5R2	80.0	78.1	5.3	4.5	Alive	Died
PYR/ATR/2-PAM/DZM	09/24/93	7C4	95.0	96.4	7.9	5.7	Died	Died
PYR/ATR/2-PAM/DZM	09/21/93	H309	110.0	108.8	7.7	8.4	Died	Died
PYR/ATR/2-PAM/DZM	09/14/93	H300	130.0	127.9	7.0	6.6	Died	Died
PYR/ATR/2-PAM/DZM	09/03/93	7D6	160.0	158.6	7.5	4.7	Alive	Died
PYR/ATR/2-PAM/DZM	09/10/93	H612	200.0	198.7	7.5	5.7	Died	Died
PYR/ATR/2-PAM/DZM	09/07/93	7AL	260.0	258.9	6.9	6.7	Died	Died

* GD doses calculated using weight losses of syringes and chemical analysis of dosing solution.

TABLE D-11. DATA LISTING FOR ADDITIONAL GROUP OF ANIMALS INJECTED WHILE RESTRAINED IN CAGES AND TREATED WITH ATR/2-PAM

Treatment Group	Date	Animal	Target GD Dose ($\mu\text{g/kg}$)	Calculated* GD Dose ($\mu\text{g/kg}$)	Body Weight (kg)	Baseline AChE Activity (U/mL)	48-Hour Results	10-Day Results
ATR/2-PAM (Cage)	10/26/93	5WT	20.5	20.1	7.7	8.5	Died	Died
ATR-2-PAM (Cage)	10/26/93	I438	20.5	20.6	7.8	9.7	Died	Died
ATR-2-PAM (Cage)	10/26/93	74H	20.5	20.6	7.3	11.9	Died	Died
ATR-2-PAM (Cage)	10/26/93	7C9	20.5	20.6	6.2	8.7	Died	Died
ATR-2-PAM (Cage)	10/26/93	6W8	20.5	20.7	7.8	10.8	Alive	Died

* GD doses calculated using weight losses of syringes and chemical analysis of dosing solution.

TABLE D-12. DATA LISTING OF CLINICAL SIGN ENDPOINTS FOR PHASE I

Treatment Group	Calculated GD Dose ^(a) ($\mu\text{g/kg}$)	Animal	Clinical Sign	Time to onset (hr)	Time to last obs. (hr)	Duration of sign in 1st 2 hr (hr)	Duration of sign in 1st 6 hr (hr)
ATR/2-PAM	9.1	H489	Appears Normal	18.00	240.00	0.00	0.00
			Tremors	0.00	6.00	1.25	5.25
			Convulsions	^(b)	-	0.00	0.00
			Salivation ^(c)	-	-	0.00	0.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	12.00	2.00	6.00
			Prostration	0.23	1.00	0.77	0.77
			Death	-	-	-	-
ATR/2-PAM	14.9	H486	Appears Normal	120.00	240.00	0.00	0.00
			Tremors	0.00	96.00	2.00	2.00
			Convulsions	-	-	0.00	0.00
			Salivation	0.00	12.00	0.50	3.50
			Miosis	-	-	0.00	0.00
			Mydriasis	0.25	12.00	1.75	5.75
			Prostration	0.30	48.00	1.70	3.70
			Death	-	-	-	-
ATR/2-PAM	15.6	G708	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	1.25	1.25	1.25
			Convulsions	0.38	1.25	0.87	0.87
			Salivation	-	-	0.00	0.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	1.28	1.28	1.28
			Prostration	0.15	1.28	1.13	1.13
			Death	1.28	-	-	-

TABLE D-12.
(Continued)

Treatment Group	Calculated GD Dose ^(a) ($\mu\text{g/kg}$)	Animal	Clinical Sign	Time to onset (hr)	Time to last obs. (hr)	Duration of sign in 1st 2 hr (hr)	Duration of sign in 1st 6 hr (hr)
ATR/2-PAM	17.0	7D4	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	96.00	1.75	5.75
			Convulsions	-	-	0.00	0.00
			Salivation	0.00	96.00	2.00	6.00
			Miosis	4.00	12.00	0.00	2.00
			Mydriasis	0.00	4.00	2.00	4.00
			Prostration	0.03	96.00	1.97	5.97
			Death	-	-	-	-
ATR/2-PAM	18.1	79C	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	72.00	2.00	4.00
			Convulsions	0.10	0.25	0.15	0.15
			Salivation	0.25	96.00	1.50	5.50
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	12.00	2.00	6.00
			Prostration	0.08	12.00	1.92	5.92
			Death	-	-	-	-
ATR/2-PAM	20.3	7BY	Appears Normal	192.00	240.00	0.00	0.00
			Tremors	0.00	192.00	2.00	6.00
			Convulsions	-	-	0.00	0.00
			Salivation	1.75	6.00	0.25	4.25
			Miosis	-	-	0.00	0.00
			Mydriasis	0.25	12.00	1.75	5.75
			Prostration	0.37	48.00	1.63	5.63
			Death	-	-	-	-

TABLE D-12.
(Continued)

Treatment Group	Calculated GD Dose ^(a) ($\mu\text{g/kg}$)	Animal	Clinical Sign	Time to onset (hr)	Time to last obs. (hr)	Duration of sign in 1st 2 hr (hr)	Duration of sign in 1st 6 hr (hr)
ATR/2-PAM	21.7	6NL	Appears Normal	48.00	72.00	0.00	0.00
			Tremors	0.00	72.00	1.75	1.75
			Convulsions	0.05	0.25	0.20	0.20
			Salivation	0.25	101.67	1.75	5.75
			Miosis	72.00	101.67	0.00	0.00
			Mydriasis	0.00	6.00	2.00	6.00
			Prostration	0.07	101.67	1.93	5.93
			Death	101.67	-	-	-
ATR/2-PAM	23.1	G757	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	1.15	0.90	0.90
			Convulsions	0.05	0.25	0.20	0.20
			Salivation	-	-	0.00	0.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	1.15	1.15	1.15
			Prostration	0.07	1.15	1.08	1.08
			Death	1.15	-	-	-
ATR/2-PAM	24.9	H263	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	0.25	0.25	0.25
			Convulsions	-	-	0.00	0.00
			Salivation	-	-	0.00	0.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	0.25	0.25	0.25
			Prostration	0.07	0.37	0.30	0.30
			Death	0.37	-	-	-

TABLE D-12.
(Continued)

Treatment Group	Calculated GD Dose ^(a) ($\mu\text{g/kg}$)	Animal	Clinical Sign	Time to onset (hr)	Time to last obs. (hr)	Duration of sign in 1st 2 hr (hr)	Duration of sign in 1st 6 hr (hr)
ATR/2-PAM	26.8	7BM	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	12.00	2.00	6.00
			Convulsions	0.02	3.00	0.23	1.23
			Salivation	0.00	12.00	1.25	5.25
			Miosis	0.00	12.00	0.50	3.50
			Mydriasis	0.50	3.00	1.50	2.50
			Prostration	0.03	12.00	1.97	5.97
			Death	20.43	-	-	-
Untreated	3.4	G867	Appears Normal	0.00	240.00	2.00	6.00
			Tremors	-	-	0.00	0.00
			Convulsions	-	-	0.00	0.00
			Salivation	-	-	0.00	0.00
			Miosis	-	-	0.00	0.00
			Mydriasis	-	-	0.00	0.00
			Prostration	-	-	0.00	0.00
			Death	-	-	-	-
Untreated	5.2	6TM	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	12.00	1.25	5.25
			Convulsions	0.27	4.00	0.98	1.98
			Salivation	0.00	30.10	2.00	6.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	30.10	2.00	6.00
			Prostration	0.25	30.10	1.75	5.75
			Death	30.10	-	-	-

TABLE D-12.
(Continued)

Treatment Group	Calculated GD Dose ^(a) ($\mu\text{g/kg}$)	Animal	Clinical Sign	Time to onset (hr)	Time to last obs. (hr)	Duration of sign in 1st 2 hr (hr)	Duration of sign in 1st 6 hr (hr)
Untreated	5.4	H444	Appears Normal	4.00	240.00	0.00	2.00
			Tremors	0.00	1.00	1.00	1.00
			Convulsions	-	-	0.00	0.00
			Salivation	-	-	0.00	0.00
			Miosis	0.25	4.00	1.75	3.75
			Mydriasis	-	-	0.00	0.00
			Prostration	-	-	0.00	0.00
			Death	-	-	-	-
Untreated	5.7	H413	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	48.00	2.00	6.00
			Convulsions	0.18	6.00	1.07	5.07
			Salivation	0.00	48.00	2.00	6.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	6.00	0.75	4.75
			Prostration	0.23	92.65	1.77	5.77
			Death	92.65	-	-	-
Untreated	6.9	77L	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	6.00	2.00	6.00
			Convulsions	0.12	3.00	1.63	2.63
			Salivation	0.00	12.00	2.00	6.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	12.00	2.00	6.00
			Prostration	0.13	12.00	1.87	5.87
			Death	20.35	-	-	-

TABLE D-12.
(Continued)

Treatment Group	Calculated GD Dose ^(a) ($\mu\text{g/kg}$)	Animal	Clinical Sign	Time to onset (hr)	Time to last obs. (hr)	Duration of sign in 1st 2 hr (hr)	Duration of sign in 1st 6 hr (hr)
Untreated	7.8	7CG	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	0.75	0.75	0.75
			Convulsions	0.08	0.25	0.17	0.17
			Salivation	0.00	0.93	0.93	0.93
			Miosis	0.25	0.50	0.25	0.25
			Mydriasis	0.00	0.75	0.50	0.50
			Prostration	0.10	0.93	0.83	0.83
			Death	0.93	-	-	-

^(a) GD doses calculated from weight losses of syringes and chemical analysis of dosing solution.

^(b) Sign was not noted during duration of the experiment.

^(c) Excessive secretion of saliva or bronchial fluids as evidenced by discharge from the mouth or nares.

TABLE D-13. DESCRIPTIVE STATISTICS OF CLINICAL SIGNS FOR THE ATR/2-PAM TREATED AND UNTREATED GROUPS OF ANIMALS IN PHASE I EXPERIMENTS BASED ON NONMISSING UNCENSORED ENDPOINTS

Clinical Sign	Endpoint	Group					
		ATR/2-PAM			Untreated		
		N ^(a)	Mean (hr)	(S.D.)	N ^(a)	Mean (hr)	(S.D.)
Tremors	Time to onset	10	0.00	(0.00)	5	0.00	(0.00)
	Duration in 1st 2 hrs	10	1.52	(0.59)	6	1.17	(0.77)
	Duration in 1st 6 hrs	10	3.31	(2.31)	6	3.17	(2.86)
Convulsions	Time to onset	5	0.12	(0.15)	4	0.16	(0.08)
	Duration in 1st 2 hrs	10	0.16	(0.27)	6	0.64	(0.68)
	Duration in 1st 6 hrs	10	0.27	(0.43)	6	1.64	(2.02)
Salivation ^(b)	Time to onset	6	0.38	(0.68)	4	0.00	(0.00)
	Duration in 1st 2 hrs	10	0.72	(0.81)	6	1.16	(0.99)
	Duration in 1st 6 hrs	10	3.02	(2.70)	6	3.16	(3.13)
Miosis	Time to onset	3	25.33	(40.46)	2	0.25	(0.00)
	Duration in 1st 2 hrs	10	0.05	(0.16)	6	0.33	(0.70)
	Duration in 1st 6 hrs	10	0.55	(1.21)	6	0.67	(1.51)
Mydriasis	Time to onset	10	0.10	(0.17)	4	0.00	(0.00)
	Duration in 1st 2 hrs	10	1.57	(0.56)	6	0.88	(0.92)
	Duration in 1st 6 hrs	10	3.87	(2.35)	6	2.87	(3.01)
Prostration	Time to onset	10	0.14	(0.12)	4	0.18	(0.07)
	Duration in 1st 2 hrs	10	1.44	(0.59)	6	1.04	(0.89)
	Duration in 1st 6 hrs	10	3.64	(2.53)	6	3.04	(3.04)
Death	Time to death ^(c)	4	5.81	(9.76)	3	17.13	(14.85)

(a) For times to onset and death, N is the number of animals that responded. For duration, N is the number of animals in study groups.

(b) Excessive secretion of saliva or bronchial fluid.

(c) Based on 48-hr endpoint.

TABLE D-14. SUMMARY OF STATISTICAL COMPARISONS BETWEEN CLINICAL SIGNS FOR THE ATR/2-PAM TREATED AND UNTREATED CONTROL GROUP FOR PHASE I EXPERIMENTS

Clinical Sign	Analyses of Variance Results Using Censored Values											
	Incidence of Clinical Sign			Fisher's Exact Test	Endpoint	Wilcoxon Test p-value	ATR/2-PAM Predicted		Untreated Predicted		Chi-Square p-value	Log-Dose Slope p-value
	ATR/2-PAM	Untreated	Mean				(S.E.) (hr)	Mean	(S.E.) (hr)			
Tremors	10/10	5/6	0.375	Time to onset	-	0.01	(0.01)	0.03	(0.03)	0.231	0.035*	
				Duration, 1st 2 hrs	0.371	1.60	(0.20)	1.17	(0.25)	0.176	-	
				Duration, 1st 6 hrs	0.785	3.64	(0.76)	3.17	(0.95)	0.697	-	
Convulsions	5/10	4/6	0.633	Time to onset	-	2.95	(4.62)	0.54	(1.04)	0.007*	0.006*	
				Duration, 1st 2 hrs	0.213	0.17	(0.14)	0.64	(0.18)	0.032*	-	
				Duration, 1st 6 hrs	0.213	0.27	(0.37)	1.64	(0.48)	0.023*	-	
Salivation/	6/10	4/6	1.000	Time to onset	-	0.43	(0.68)	0.13	(0.26)	0.027*	0.022*	
Bronchial				Duration, 1st 2 hrs	0.340	0.73	(0.27)	1.29	(0.36)	0.204	-	
Secretions				Duration, 1st 6 hrs	0.466	3.03	(0.84)	3.74	(1.16)	0.616	-	
Miosis	3/10	2/6	1.000	Time to onset	-	158	(354)	25.3	(64.3)	0.044*	0.041*	
				Duration, 1st 2 hrs	0.265	0.05	(0.13)	0.33	(0.17)	0.180	-	
				Duration, 1st 6 hrs	0.569	0.55	(0.39)	0.67	(0.51)	0.856	-	
Mydriasis	10/10	4/6	0.125	Time to onset	-	0.02	(0.02)	0.26	(0.40)	0.671	0.216	
				Duration, 1st 2 hrs	0.181	1.72	(0.23)	0.88	(0.28)	0.019*	-	
				Duration, 1st 6 hrs	0.440	4.62	(0.80)	2.88	(0.95)	0.159	-	
Prostration	10/10	4/6	0.125	Time to onset	-	0.06	(0.04)	1.25	(1.16)	0.375	0.016*	
				Duration, 1st 2 hrs	0.277	1.75	(0.23)	1.14	(0.27)	0.080	-	
				Duration, 1st 6 hrs	0.277	4.92	(0.82)	3.57	(0.97)	0.290	-	
Death	4/10	3/6	1.000	Time to death	-	33.4	(36.9)	27.6	(36.7)	0.061	0.041*	
Explanation of column headings												

Explanation of column headings

Incidence of Clinical Sign: Proportion of animals in each group that exhibited the clinical sign.

Fisher's Exact Test: P-value for comparing the incidence of a clinical sign between the ATR/2-PAM group and untreated control group.

Wilcoxon Test: P-value for Wilcoxon's rank sum test comparing nonmissing, uncensored durations between the ATR/2-PAM and untreated control groups. This test was not performed on times to onset since they were determined to be dose-dependent.

Analyses of Variance Results Using Censored Values:

Predicted Mean: The mean value of endpoint predicted by the ANOVA for each group. S.E. is the standard error of the predicted mean.

Predicted means were computed at the GD 48 hr LD₅₀ for each group, 20.5 µg/kg and 6.50 µg/kg for the ATR/2-PAM group and untreated group, respectively.

Chi-square p-value: P-value comparing the mean predicted values between the ATR/2-PAM and untreated control groups.

Log-Dose Slope p-value: P-value for the log GD dose slope. P-values for the log-dose slope were nonsignificant for durations within 2 hr and 6 hr, so the log GD dose covariate was dropped from the model for duration.

* Statistically significant at the 0.05 level.

TABLE D-15. DATA LISTING OF CLINICAL SIGN ENDPOINTS FOR PHASE III

PYR Dose ($\mu\text{g/kg i.m.}$)	Animal	Date	Clinical Sign	Time to onset (hr)	Time to last obs. (hr)	Duration of sign in 1st 2 hr (hr)	Duration of sign in 1st 6 hr (hr)
0.0	75G	04/20/93	Appears Normal	-(a)	-	0.00	0.00
			Tremors	0.00	0.75	0.75	0.75
			Convulsions	0.45	0.92	0.47	0.47
			Salivation ^(b)	0.00	0.92	0.92	0.92
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	0.92	0.92	0.92
			Prostration	0.03	0.92	0.88	0.88
			Death	0.92	-	-	-
			Appears Normal	-	-	0.00	0.00
0.0	78J	04/20/93	Tremors	0.00	0.25	0.25	0.25
			Convulsions	-	-	0.00	0.00
			Salivation	0.00	2.42	2.00	2.42
			Miosis	0.00	0.50	0.50	0.50
			Mydriasis	0.00	0.25	0.25	0.25
			Prostration	0.05	2.42	1.95	2.37
			Death	2.42	-	-	-
			Appears Normal	-	-	0.00	0.00
			Tremors	0.00	23.97	2.00	6.00
0.0	7CA	05/25/93	Convulsions	-	-	0.00	0.00
			Salivation	0.00	23.97	0.75	4.75
			Miosis	0.25	23.97	1.75	5.75
			Mydriasis	0.00	0.50	0.50	0.50
			Prostration	0.03	23.97	1.97	5.97
			Death	23.97	-	-	-
			Appears Normal	-	-	0.00	0.00
			Tremors	0.00	0.50	0.50	0.50
			Convulsions	0.12	0.50	0.38	0.38
0.0	H355	05/25/93	Salivation	0.00	0.63	0.63	0.63
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	0.63	0.63	0.63
			Prostration	0.05	0.50	0.45	0.45
			Death	0.63	-	-	-
			Appears Normal	144.00	240.00	0.00	0.00
			Tremors	0.00	96.00	2.00	5.50
			Convulsions	0.18	0.25	0.07	0.07
			Salivation	0.50	0.75	0.25	0.25
4.0	6RA	04/20/93	Miosis	-	-	0.00	0.00
			Mydriasis	0.00	12.00	2.00	6.00
			Prostration	0.08	12.00	1.92	5.42
			Death	-	-	-	-
			Appears Normal	-	-	0.00	0.00
			Tremors	0.00	96.00	1.50	5.50
			Convulsions	-	-	0.00	0.00
			Salivation	0.25	72.00	1.00	1.00
			Miosis	18.00	240.00	0.00	0.00
4.0	79Y	04/20/93	Mydriasis	0.00	0.50	0.50	0.50
			Prostration	0.03	6.00	1.97	4.47
			Death	-	-	-	-
			Appears Normal	-	-	0.00	0.00
			Tremors	0.00	96.00	1.50	5.50
			Convulsions	-	-	0.00	0.00

TABLE D-15.
(Continued)

PYR Dose ($\mu\text{g/kg i.m.}$)	Animal	Date	Clinical Sign	Time to onset (hr)	Time to last obs. (hr)	Duration of sign in 1st 2 hr (hr)	Duration of sign in 1st 6 hr (hr)
4.0	G654	04/27/93	Appears Normal	48.00	240.00	0.00	0.00
			Tremors	0.00	3.00	1.25	2.25
			Convulsions	0.05	0.25	0.20	0.20
			Salivation	3.00	12.00	0.00	3.00
			Miosis	24.00	48.00	0.00	0.00
			Mydriasis	0.00	4.50	2.00	4.50
			Prostration	0.05	12.00	1.95	5.95
			Death	-	-	-	-
4.0	H831	04/27/93	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	0.82	0.82	0.82
			Convulsions	0.10	0.82	0.72	0.72
			Salivation	0.00	0.50	0.50	0.50
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	0.82	0.82	0.82
			Prostration	0.05	0.82	0.77	0.77
			Death	0.82	-	-	-
4.0	78X	05/04/93	Appears Normal	120.00	144.00	0.00	0.00
			Tremors	0.00	1.25	1.25	1.25
			Convulsions	0.13	1.25	1.12	1.12
			Salivation	0.00	48.00	2.00	6.00
			Miosis	0.75	240.00	0.75	4.75
			Mydriasis	0.00	0.75	0.75	0.75
			Prostration	0.05	12.00	1.95	5.95
			Death	-	-	-	-
4.0	H818	05/04/93	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	72.00	2.00	6.00
			Convulsions	0.63	4.00	0.62	1.62
			Salivation	0.50	96.00	1.50	5.50
			Miosis	24.00	72.00	0.00	0.00
			Mydriasis	0.00	6.00	2.00	6.00
			Prostration	0.05	96.00	1.95	5.95
			Death	-	-	-	-
4.0	6WB	05/18/93	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	192.00	1.75	5.25
			Convulsions	4.10	4.50	0.00	0.40
			Salivation	0.00	2.00	0.50	0.50
			Miosis	2.50	216.00	0.00	3.50
			Mydriasis	0.00	1.00	1.00	1.00
			Prostration	0.03	12.00	1.97	4.97
			Death	-	-	-	-
4.0	75F	05/18/93	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	216.00	1.00	4.50
			Convulsions	0.15	1.50	1.35	1.35
			Salivation	0.00	48.00	1.50	5.00
			Miosis	120.00	216.00	0.00	0.00
			Mydriasis	0.00	3.50	1.75	3.25
			Prostration	0.03	12.00	1.97	5.97
			Death	-	-	-	-

TABLE D-15.
(Continued)

PYR Dose ($\mu\text{g/kg i.m.}$)	Animal	Date	Clinical Sign	Time to onset (hr)	Time to last obs. (hr)	Duration of sign in 1st 2 hr (hr)	Duration of sign in 1st 6 hr (hr)
4.0	76P	05/25/93	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	1.35	1.35	1.35
			Convulsions	0.40	1.25	0.60	0.60
			Salivation	0.25	1.25	1.00	1.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	1.35	1.35	1.35
			Prostration	0.05	1.35	1.30	1.30
			Death	1.35	-	-	-
4.0	7AH	06/08/93	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	1.50	1.50	1.50
			Convulsions	0.12	1.25	0.88	0.88
			Salivation	0.75	54.83	0.75	4.75
			Miosis	48.00	54.83	0.00	0.00
			Mydriasis	0.00	48.00	2.00	6.00
			Prostration	0.13	54.83	1.87	5.87
			Death	54.83	-	-	-
8.4	77K	04/06/93	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	1.25	1.25	1.25
			Convulsions	0.05	0.25	0.20	0.20
			Salivation	0.00	12.00	2.00	6.00
			Miosis	18.00	216.00	0.00	0.00
			Mydriasis	0.00	1.00	1.00	1.00
			Prostration	0.07	12.00	1.93	5.93
			Death	-	-	-	-
8.4	G244	04/06/93	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	72.00	1.50	5.50
			Convulsions	0.03	12.00	0.22	1.72
			Salivation	0.25	96.00	1.25	3.75
			Miosis	18.00	24.00	0.00	0.00
			Mydriasis	0.00	12.00	2.00	4.50
			Prostration	0.08	96.00	1.92	5.92
			Death	-	-	-	-
8.4	6Z1	04/13/93	Appears Normal	144.00	240.00	0.00	0.00
			Tremors	0.00	5.50	2.00	4.50
			Convulsions	-	-	0.00	0.00
			Salivation	0.00	1.50	1.50	1.50
			Miosis	0.00	72.00	2.00	6.00
			Mydriasis	0.00	0.25	0.25	0.25
			Prostration	0.05	1.00	0.95	0.95
			Death	-	-	-	-
8.4	78Y	04/13/93	Appears Normal	144.00	240.00	0.00	0.00
			Tremors	0.00	3.50	1.75	3.25
			Convulsions	-	-	0.00	0.00
			Salivation	0.00	72.00	1.25	1.25
			Miosis	1.25	72.00	0.75	4.75
			Mydriasis	0.00	0.25	0.25	0.25
			Prostration	0.03	1.25	1.22	1.22
			Death	-	-	-	-

TABLE D-15.
(Continued)

PYR Dose ($\mu\text{g/kg i.m.}$)	Animal	Date	Clinical Sign	Time to onset (hr)	Time to last obs. (hr)	Duration of sign in 1st 2 hr (hr)	Duration of sign in 1st 6 hr (hr)
8.4	6PJ	04/27/93	Appears Normal	48.00	240.00	0.00	0.00
			Tremors	0.00	12.00	2.00	6.00
			Convulsions	0.07	1.00	0.93	0.93
			Salivation	0.25	12.00	1.00	5.00
			Miosis	1.00	48.00	0.50	1.00
			Mydriasis	0.00	1.00	1.00	1.00
			Prostration	0.03	12.00	1.97	5.97
			Death	-	-	-	-
8.4	H436	04/27/93	Appears Normal	96.00	240.00	0.00	0.00
			Tremors	0.00	12.00	1.75	5.75
			Convulsions	-	-	0.00	0.00
			Salivation	-	-	0.00	0.00
			Miosis	5.00	72.00	0.00	1.00
			Mydriasis	0.00	1.25	1.25	1.25
			Prostration	0.05	48.00	1.20	1.20
			Death	-	-	-	-
8.4	6W2	05/18/93	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	240.00	1.25	4.75
			Convulsions	5.50	12.00	0.00	0.50
			Salivation	0.00	48.00	0.75	0.75
			Miosis	3.00	216.00	0.00	3.00
			Mydriasis	0.00	0.25	0.25	0.25
			Prostration	0.05	2.00	1.95	1.95
			Death	-	-	-	-
8.4	H602	05/18/93	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	144.00	0.25	3.25
			Convulsions	-	-	0.00	0.00
			Salivation	0.25	0.50	0.25	0.25
			Miosis	18.00	240.00	0.00	0.00
			Mydriasis	0.00	4.50	2.00	4.50
			Prostration	0.03	12.00	1.97	5.97
			Death	-	-	-	-
8.4	H432	05/25/93	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	0.87	0.87	0.87
			Convulsions	0.23	0.50	0.27	0.27
			Salivation	0.00	0.75	0.75	0.75
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	0.87	0.87	0.87
			Prostration	0.05	0.75	0.70	0.70
			Death	0.87	-	-	-
8.4	6XR	06/08/93	Appears Normal	96.00	240.00	0.00	0.00
			Tremors	0.00	5.50	1.50	4.00
			Convulsions	-	-	0.00	0.00
			Salivation	0.00	1.50	1.00	1.00
			Miosis	1.75	96.00	0.25	4.25
			Mydriasis	0.00	1.25	1.25	1.25
			Prostration	0.05	3.00	1.45	1.95
			Death	-	-	-	-

TABLE D-15.
(Continued)

PYR Dose ($\mu\text{g/kg i.m.}$)	Animal	Date	Clinical Sign	Time to onset (hr)	Time to last obs. (hr)	Duration of sign in 1st 2 hr (hr)	Duration of sign in 1st 6 hr (hr)
24.0	77V	04/06/93	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	4.00	2.00	4.00
			Convulsions	0.88	1.00	0.12	0.12
			Salivation	0.25	45.58	1.75	5.75
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	4.00	2.00	4.00
			Prostration	0.05	45.58	1.95	5.95
			Death	45.58	-	-	-
24.0	H632	04/06/93	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	2.50	2.00	2.50
			Convulsions	0.02	2.50	0.73	1.23
			Salivation	1.25	12.00	0.75	4.75
			Miosis	18.00	141.67	0.00	0.00
			Mydriasis	0.00	2.50	2.00	2.50
			Prostration	0.07	12.00	1.93	5.93
			Death	141.67	-	-	-
24.0	6XC	04/13/93	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	5.50	1.75	4.25
			Convulsions	0.12	5.00	0.88	1.38
			Salivation	0.00	43.33	2.00	6.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	43.33	2.00	6.00
			Prostration	0.02	43.33	1.98	5.98
			Death	43.33	-	-	-
24.0	H227	04/13/93	Appears Normal	24.00	240.00	0.00	0.00
			Tremors	0.00	6.00	2.00	4.50
			Convulsions	-	-	0.00	0.00
			Salivation	0.00	0.75	0.75	0.75
			Miosis	1.75	5.00	0.25	3.25
			Mydriasis	0.00	1.00	1.00	1.00
			Prostration	0.03	1.00	0.97	0.97
			Death	-	-	-	-
24.0	71D	05/04/93	Appears Normal	120.00	144.00	0.00	0.00
			Tremors	0.00	12.00	2.00	5.50
			Convulsions	0.08	0.25	0.17	0.17
			Salivation	-	-	0.00	0.00
			Miosis	2.00	240.00	0.00	2.50
			Mydriasis	0.00	0.50	0.50	0.50
			Prostration	0.07	1.25	1.18	1.18
			Death	-	-	-	-
24.0	H789	05/04/93	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	68.25	1.75	3.25
			Convulsions	0.15	1.75	1.60	1.60
			Salivation	0.00	68.25	1.50	2.00
			Miosis	5.00	68.25	0.00	1.00
			Mydriasis	0.00	68.25	1.25	1.75
			Prostration	0.03	68.25	1.97	5.97
			Death	68.25	-	-	-

TABLE D-15.
(Continued)

PYR Dose ($\mu\text{g/kg i.m.}$)	Animal	Date	Clinical Sign	Time to onset (hr)	Time to last obs. (hr)	Duration of sign in 1st 2 hr (hr)	Duration of sign in 1st 6 hr (hr)
24.0	78S	05/18/93	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	48.00	2.00	6.00
			Convulsions	0.07	2.50	1.93	2.43
			Salivation	0.75	48.00	1.25	5.25
			Miosis	0.75	96.00	0.25	0.25
			Mydriasis	0.00	5.00	1.50	4.50
			Prostration	0.03	96.00	1.97	5.97
			Death	-	-	-	-
24.0	H282	05/18/93	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	72.00	1.25	4.75
			Convulsions	0.05	0.25	0.20	0.20
			Salivation	-	-	0.00	0.00
			Miosis	0.75	240.00	1.25	5.25
			Mydriasis	0.00	0.50	0.50	0.50
			Prostration	0.05	1.25	1.20	1.20
			Death	-	-	-	-
24.0	H816	05/25/93	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	0.88	0.88	0.88
			Convulsions	-	-	0.00	0.00
			Salivation	0.25	0.88	0.63	0.63
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	0.88	0.88	0.88
			Prostration	0.05	0.88	0.83	0.83
			Death	0.88	-	-	-
24.0	H483	06/08/93	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	168.00	0.50	3.50
			Convulsions	0.05	0.25	0.20	0.20
			Salivation	0.00	12.00	2.00	6.00
			Miosis	18.00	240.00	0.00	0.00
			Mydriasis	0.00	4.00	2.00	4.00
			Prostration	0.05	3.50	1.95	3.45
			Death	-	-	-	-

(a) Sign was not noted during duration of the experiment.

(b) Excessive secretion of saliva or bronchial fluids.

TABLE D-16. DESCRIPTIVE STATISTICS OF CLINICAL SIGNS FOR THE CONTROL GROUP AND PYR-DOSED GROUPS IN PHASE III EXPERIMENTS BASED ON NONMISSING UNCENSORED ENDPOINTS

Clinical Sign	Endpoint	Untreated Control						PYR Dose Group					
		4 $\mu\text{g/kg}$			8.4 $\mu\text{g/kg}$			24 $\mu\text{g/kg}$			4-24 $\mu\text{g/kg}^{(a)}$		
		N ^(b)	Mean (hr)	(S.D.)	N	Mean (hr)	(S.D.)	N	Mean (hr)	(S.D.)	N	Mean (hr)	(S.D.)
Tremors	Time to onset	4	0.00	(0.00)	10	0.00	(0.00)	10	0.00	(0.00)	30	0.00	(0.00)
	Duration in 1st 2 hrs	4	0.88	(0.78)	10	1.44	(0.39)	10	1.41	(0.54)	30	1.49	(0.49)
	Duration in 1st 6 hrs	4	1.87	(2.76)	10	3.39	(2.13)	10	3.91	(1.78)	30	3.74	(1.77)
Convulsions	Time to onset	2	0.28	(0.24)	9	0.65	(1.31)	5	1.18	(2.42)	8	0.18	(0.29)
	Duration in 1st 2 hrs	4	0.21	(0.25)	10	0.56	(0.48)	10	0.16	(0.29)	10	0.58	(0.69)
	Duration in 1st 6 hrs	4	0.21	(0.25)	10	0.70	(0.55)	10	0.36	(0.56)	10	0.73	(0.86)
Salivation/ Bronchial Secretions	Time to onset	4	0.00	(0.00)	10	0.53	(0.91)	9	0.08	(0.12)	8	0.31	(0.46)
	Duration in 1st 2 hrs	4	1.07	(0.63)	7	0.90	(0.63)	10	0.97	(0.58)	10	1.06	(0.75)
	Duration in 1st 6 hrs	4	2.18	(1.88)	10	2.75	(2.35)	10	2.02	(2.11)	10	3.11	(2.65)
Miosis	Time to onset	2	0.13	(0.18)	10	33.89	(41.12)	9	7.33	(8.12)	7	6.61	(7.91)
	Duration in 1st 2 hrs	4	0.56	(0.83)	10	0.08	(0.24)	10	0.35	(0.64)	10	0.17	(0.39)
	Duration in 1st 6 hrs	4	1.56	(2.80)	10	0.82	(1.76)	10	2.00	(2.30)	10	1.22	(1.84)
Mydriasis	Time to onset	4	0.00	(0.00)	10	0.00	(0.00)	10	0.00	(0.00)	10	0.00	(0.00)
	Duration in 1st 2 hrs	4	0.57	(0.28)	10	1.42	(0.61)	10	1.01	(0.65)	10	1.36	(0.62)
	Duration in 1st 6 hrs	4	0.57	(0.28)	10	3.02	(2.41)	10	1.51	(1.62)	10	2.56	(1.95)
Prostration	Time to onset	4	0.04	(0.01)	10	0.06	(0.03)	10	0.05	(0.02)	10	0.04	(0.02)
	Duration in 1st 2 hrs	4	1.31	(0.77)	10	1.76	(0.40)	10	1.52	(0.48)	10	1.59	(0.48)
	Duration in 1st 6 hrs	4	2.42	(2.50)	10	4.66	(1.98)	10	3.17	(2.42)	10	3.74	(2.45)
Death	Time to death ^(c)	4	6.98	(11.35)	2	1.08	(0.38)	1	0.87	(-)	3	29.93	(25.18)

^(a) Descriptive statistics for all three PYR dose groups pooled.

^(b) For times to onset and death, N is the number of animals that responded. For durations, N is the number of animals in the study groups.

^(c) Based on 48-hr endpoint.

TABLE D-17. SUMMARY OF STATISTICAL COMPARISONS BETWEEN CLINICAL SIGNS FOR THE CONTROL GROUP AND POOLED PYR-DOSED GROUPS FOR PHASE III EXPERIMENTS

Incidence of Clinical Sign		Analyses of Variance Results Using Censored Values								Chi-Square p-value		
		Clinical Sign	Control	4-24 $\mu\text{g/kg}$	Fisher's Exact Test	Endpoint	Wilcoxon Test p-value	Control Group Predicted			4-24 $\mu\text{g/kg}$ Predicted	
								Mean	(S.E.)		Mean	(S.E.)
								(hr)		(hr)		
Tremors	4/4	30/30	1.000	Time to onset	1.000	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)	1.000
				Duration in 1st 2 hrs	0.097	0.88	(0.24)	1.59	(0.09)	0.007*		
				Duration in 1st 6 hrs	0.092	1.87	(0.80)	4.18	(0.31)	0.007*		
Convulsions	2/4	22/30	0.564	Time to onset	0.463	7.40	(17.71)	1.67	(1.37)	0.554		
				Duration in 1st 2 hrs	0.511	0.30	(0.27)	0.44	(0.10)	0.621		
				Duration in 1st 6 hrs	0.303	0.33	(0.34)	0.61	(0.12)	0.431		
Salivation/ Bronchial Secretions	4/4	27/30	1.000	Time to onset	0.085	0.01	(0.02)	0.16	(0.09)	0.102		
				Duration in 1st 2 hrs	0.914	1.52	(0.39)	1.00	(0.12)	0.201		
				Duration in 1st 6 hrs	0.810	5.39	(1.86)	2.72	(0.41)	0.161		
Miosis	2/4	23/30	0.281	Time to onset	0.030*	0.45	(0.56)	7.93	(3.54)	0.031*		
				Duration in 1st 2 hrs	0.273	0.56	(0.24)	0.20	(0.09)	0.160		
				Duration in 1st 6 hrs	0.814	1.56	(1.00)	1.35	(0.37)	0.842		
Mydriasis	4/4	30/30	1.000	Time to onset	1.000	0.00	(0.00)	0.00	(0.00)	1.000		
				Duration in 1st 2 hrs	0.039*	0.84	(0.35)	1.35	(0.12)	0.169		
				Duration in 1st 6 hrs	0.041*	1.52	(1.13)	2.68	(0.38)	0.327		
Prostration	4/4	30/30	1.000	Time to onset	0.388	0.04	(0.01)	0.05	(0.00)	0.468		
				Duration in 1st 2 hrs	0.499	1.48	(0.24)	1.71	(0.08)	0.378		
				Duration in 1st 6 hrs	0.321	3.85	(1.32)	4.21	(0.41)	0.789		
Death	4/4	6/30	0.005*	Time to death	0.831	2.41	(3.97)	575	(689)	0.007*		

Explanation of column headings

Incidence of Clinical Sign: Proportion of animals in each group that exhibited the clinical sign.

4-24 $\mu\text{g/kg}$: Proportion over all three PYR dose groups (4 $\mu\text{g/kg}$, 8.4 $\mu\text{g/kg}$, 24 $\mu\text{g/kg}$).

Fisher's Exact Test: p-value for comparing the incidence of clinical signs between the untreated control group and the pooled 4-24 $\mu\text{g/kg}$ PYR group.

Wilcoxon Test: p-value for Wilcoxon's rank sum test comparing nonmissing, uncensored durations between the untreated control group and the pooled 4-24 $\mu\text{g/kg}$ PYR group.

Analyses of Variance Using Censored Values:

Predicted Mean: The mean value of endpoint predicted by the ANOVA for each group. S.E. is the standard error of the predicted mean.

Chi-square p-value: p-value for comparing the mean predicted values between the untreated control group and the pooled 4-24 $\mu\text{g/kg}$ PYR group.
* Statistically significant at the 0.05 level.

TABLE D-18. DATA LISTING OF CLINICAL SIGN ENDPOINTS FOR
PHASE IV EXPERIMENTS

Calculated ^(a) PYR Dose ($\mu\text{g/kg i.g.}$)	Animal	Date	Clinical Sign	Time to onset (hr)	Time to last obs. (hr)	Duration of sign in 1st 2 hr (hr)	Duration of sign in 1st 6 hr (hr)
40	75H	08/03/93	Appears Normal	-(b)	-	0.00	0.00
			Tremors	0.00	240.00	2.00	6.00
			Convulsions	0.05	4.00	1.45	2.95
			Salivation ^(c)	0.00	48.00	2.00	6.00
			Miosis	1.00	240.00	0.75	4.75
			Mydriasis	0.00	0.25	0.25	0.25
			Prostration	0.05	12.00	1.95	5.95
			Death	-	-	-	-
40	H167	08/03/93	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	139.58	2.00	6.00
			Convulsions	0.65	2.50	1.35	1.85
			Salivation	0.00	48.00	2.00	6.00
			Miosis	0.00	72.00	1.25	2.75
			Mydriasis	0.00	0.25	0.25	0.25
			Prostration	0.05	12.00	1.95	5.95
			Death	139.58	-	-	-
40	5U3	08/09/93	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	0.17	0.17	0.17
			Convulsions	-	-	0.00	0.00
			Salivation	-	-	0.00	0.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	0.17	0.17	0.17
			Prostration	0.05	0.17	0.12	0.12
			Death	0.17	-	-	-
40	6TR	08/09/93	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	4.50	2.00	4.50
			Convulsions	1.17	1.50	0.33	0.33
			Salivation	0.00	72.00	2.00	6.00
			Miosis	3.50	72.00	0.00	2.50
			Mydriasis	0.00	1.75	1.75	1.75
			Prostration	0.03	72.00	1.72	1.72
			Death	-	-	-	-
40	G923	08/10/93	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	96.00	1.00	1.50
			Convulsions	0.05	0.25	0.20	0.20
			Salivation	0.25	48.00	1.75	5.25
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	12.00	2.00	6.00
			Prostration	0.07	141.00	1.93	5.93
			Death	141.00	-	-	-

TABLE 18.
(Continued)

Calculated ^(a) PYR Dose ($\mu\text{g/kg i.g.}$)	Animal	Date	Clinical Sign	Time to onset (hr)	Time to last obs. (hr)	Duration of sign in 1st 2 hr (hr)	Duration of sign in 1st 6 hr (hr)
40	H843	08/10/93	Appears Normal	96.00	240.00	0.00	0.00
			Tremors	0.00	12.00	2.00	6.00
			Convulsions	0.57	1.00	0.43	0.43
			Salivation	0.00	1.50	1.50	1.50
			Miosis	18.00	96.00	0.00	0.00
			Mydriasis	0.00	12.00	2.00	5.00
			Prostration	0.05	4.50	1.70	3.20
			Death	-	-	-	-
39	7CU	08/23/93	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	4.50	2.00	4.00
			Convulsions	-	-	0.00	0.00
			Salivation	0.50	96.00	0.50	0.50
			Miosis	18.00	96.00	0.00	0.00
			Mydriasis	0.00	12.00	2.00	5.50
			Prostration	0.08	12.00	1.92	5.92
			Death	-	-	-	-
40	H237	08/23/93	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	240.00	1.75	5.75
			Convulsions	1.68	1.75	0.07	0.07
			Salivation	0.00	2.50	2.00	2.50
			Miosis	4.50	48.00	0.00	1.50
			Mydriasis	0.00	0.25	0.25	0.25
			Prostration	0.03	48.00	1.97	2.97
			Death	-	-	-	-
40	6XM	08/24/93	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	6.00	2.00	4.50
			Convulsions	-	-	0.00	0.00
			Salivation	-	-	0.00	0.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	96.00	2.00	6.00
			Prostration	0.55	72.00	1.45	5.45
			Death	-	-	-	-
39	7BK	08/24/93	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	43.83	2.00	6.00
			Convulsions	0.40	4.00	0.60	2.10
			Salivation	1.75	12.00	0.25	4.25
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	43.83	2.00	6.00
			Prostration	0.08	43.83	1.92	5.92
			Death	43.83	-	-	-

^(a) Based on weight losses of syringes and concentration analysis of dosing solution.

^(b) Sign was not noted during duration of the experiment.

^(c) Excessive salivation/bronchial secretions.

TABLE D-19. DESCRIPTIVE STATISTICS OF CLINICAL SIGNS FOR PHASE III AND PHASE IV EXPERIMENTS BASED ON NONMISSING UNCENSORED ENDPOINTS

Clinical Sign	Endpoint	PYR Dose Group								
		Group 1			Group 2			Group 3		
		Untreated Control			4-24 $\mu\text{g/kg}$ i.m. PYR ^(a)			40 $\mu\text{g/kg}$ i.g. PYR ^(b)		
		N ^(c)	Mean	(S.D.)	N	Mean	(S.D.)	N	Mean	(S.D.)
			(hr)			(hr)			(hr)	
Tremors	Time to onset	4	0.00	(0.00)	30	0.00	(0.00)	10	0.00	(0.00)
	Duration in 1st 2 hrs	4	0.88	(0.78)	30	1.49	(0.49)	10	1.69	(0.62)
	Duration in 1st 6 hrs	4	1.87	(2.76)	30	3.74	(1.77)	10	4.44	(2.07)
Convulsions	Time to onset	2	0.28	(0.24)	22	0.60	(1.39)	7	0.65	(0.59)
	Duration in 1st 2 hrs	4	0.21	(0.25)	30	0.43	(0.53)	10	0.44	(0.54)
	Duration in 1st 6 hrs	4	0.21	(0.25)	30	0.60	(0.67)	10	0.79	(1.08)
Salivation/ Bronchial Secretions	Time to onset	4	0.00	(0.00)	27	0.31	(0.62)	8	0.31	(0.61)
	Duration in 1st 2 hrs	4	1.07	(0.63)	30	0.98	(0.64)	10	1.20	(0.90)
	Duration in 1st 6 hrs	4	2.18	(1.88)	30	2.63	(2.34)	10	3.20	(2.58)
Miosis	Time to onset	2	0.13	(0.18)	23	15.20	(25.73)	6	7.50	(8.29)
	Duration in 1st 2 hrs	4	0.56	(0.83)	30	0.20	(0.45)	10	0.20	(0.44)
	Duration in 1st 6 hrs	4	1.56	(2.80)	30	1.35	(1.98)	10	1.15	(1.68)
Mydriasis	Time to onset	4	0.00	(0.00)	30	0.00	(0.00)	10	0.00	(0.00)
	Duration in 1st 2 hrs	4	0.57	(0.28)	30	1.26	(0.63)	10	1.27	(0.90)
	Duration in 1st 6 hrs	4	0.57	(0.28)	30	2.36	(2.05)	10	3.12	(2.78)
Prostration	Time to onset	4	0.04	(0.01)	30	0.05	(0.02)	10	0.11	(0.16)
	Duration in 1st 2 hrs	4	1.31	(0.77)	30	1.63	(0.45)	10	1.66	(0.57)
	Duration in 1st 6 hrs	4	2.42	(2.50)	30	3.86	(2.30)	10	4.31	(2.16)
Death	Time to death ^(d)	4	6.98	(11.35)	6	15.47	(22.46)	2	22.00	(30.88)

^(a) Pooled over the three i.m. PYR dose groups (4 $\mu\text{g/kg}$, 8.4 $\mu\text{g/kg}$, and 24 $\mu\text{g/kg}$) in Phase III.

^(b) 40 $\mu\text{g/kg}$ PYR administered i.g. in Phase IV.

^(c) For times to onset and death, N is the number of animals that responded. For durations, N is the number of animals in the study group.

^(d) Based on 48-hr endpoint.

TABLE D-20. STATISTICAL COMPARISONS BETWEEN CLINICAL SIGNS FOR PHASE III
AND PHASE IV EXPERIMENTS

Clinical Sign	Incidence of Clinical Signs			Fisher's Exact Test p-values		Wilcoxon Test p-values		Analyses of Variance Results Using Censored Values																																																																																																																																																																																																																																																																																																																																																																																																																								
	Group 1	Group 2	Group 3	Grps 1&3 Grps 2&3		Grps 1&3 Grps 2&3		Group 1		Group 2		Group 3		Chi-Square p-value																																																																																																																																																																																																																																																																																																																																																																																																																		
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Endpoint	Time to onset	1.000	1.000	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)	0.00	(0.00)	1.000	1.000																																																																																																																																																																																																																																																																																																																																																																																																																			
Tremors	4/4	30/30	10/10	1.000	1.000	1.000	1.000	0.089	0.089	0.88	(0.23)	1.58	(0.09)	1.86	(0.15)	0.000*	0.086																																																																																																																																																																																																																																																																																																																																																																																																															
Convulsions	2/4	22/30	7/10	0.580	1.000	0.193	0.149	0.556	0.192	0.563	0.911	0.899	0.563	0.181	0.746	1.000	0.404	0.570	0.586	0.129	0.288	0.789	0.754	0.974	0.802	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	0.395	1.000	0.739	1.000	0.421	0.430	0.136	0.179	0.943	

Explanation of column headings

Incidence of Clinical Sign: Proportion of animals in each group that exhibited the clinical sign.

Group definitions are: Group 1 = Phase III Control group

Group 2 = Phase III pooled 4-24 µg/kg i.m. PYR groups

Group 3 = Phase IV 40 µg/kg i.g. PYR group

Fisher's Exact Test: p-value for comparing the incidence of clinical signs between pairs of study groups.

Wilcoxon Test: p-value for Wilcoxon's rank sum test comparing nonmissing, uncensored durations between pairs of study groups.

Analyses of Variance Using Censored Values:

Predicted Mean: The mean value of endpoint predicted by the ANOVA for each group. S.E. is the standard error of the predicted mean.

Chi-square p-value: p-value for comparing the mean predicted values between pairs of groups.

* Statistically significant at the 0.05 level.

TABLE D-21. DATA LISTING OF CLINICAL SIGN ENDPOINTS FROM PHASE V

Treatment Group	Calculated GD Dose ^(a) ($\mu\text{g/kg}$)	Animal	Clinical Sign	Time to onset (hr)	Time to last obs. (hr)	Duration of sign in 1st 2 hr (hrs)	Duration of sign in 1st 6 hr (hrs)
ATR/2-PAM	5.7	H482	Appears Normal	-(b)	-	0.00	0.00
			Tremors	-	-	0.00	0.00
			Convulsions	-	-	0.00	0.00
			Salivation ^(c)	-	-	0.00	0.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	96.00	2.00	6.00
			Prostration	-	-	0.00	0.00
			Death	-	-	-	-
ATR/2-PAM	6.9	66P	Appears Normal	192.00	240.00	0.00	0.00
			Tremors	0.00	192.00	2.00	5.50
			Convulsions	-	-	0.00	0.00
			Salivation	-	-	0.00	0.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	96.00	2.00	6.00
			Prostration	-	-	0.00	0.00
			Death	-	-	-	-
ATR/2-PAM	8.5	75P	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	4.50	2.00	4.50
			Convulsions	-	-	0.00	0.00
			Salivation	-	-	0.00	0.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	168.00	2.00	6.00
			Prostration	0.18	1.25	1.07	1.07
			Death	-	-	-	-
ATR/2-PAM	7.9	7AC	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	0.72	0.72	0.72
			Convulsions	-	-	0.00	0.00
			Salivation	-	-	0.00	0.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	0.72	0.72	0.72
			Prostration	0.13	0.72	0.58	0.58
			Death	0.72	-	-	-
ATR/2-PAM	8.5	74A	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	3.50	2.00	3.50
			Convulsions	-	-	0.00	0.00
			Salivation	-	-	0.00	0.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.25	240.00	1.75	5.75
			Prostration	-	-	0.00	0.00
			Death	-	-	-	-
ATR/2-PAM	10.0	6VZ	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	0.83	0.83	0.83
			Convulsions	0.20	0.75	0.55	0.55
			Salivation	-	-	0.00	0.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	0.83	0.83	0.83
			Prostration	0.20	0.83	0.63	0.63
			Death	0.83	-	-	-

TABLE D-21.
(Continued)

Treatment Group	Calculated GD Dose ^(a) ($\mu\text{g/kg}$)	Animal	Clinical Sign	Time to onset (hr)	Time to last obs. (hr)	Duration of sign in 1st 2 hr (hrs)	Duration of sign in 1st 6 hr (hrs)
ATR/2-PAM	13.2	H258	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	0.75	0.75	0.75
			Convulsions	0.10	0.50	0.40	0.40
			Salivation	-	-	0.00	0.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	0.75	0.50	0.50
			Prostration	0.12	0.75	0.63	0.63
			Death	0.75	-	-	-
ATR/2-PAM	13.3	6RB	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	1.33	1.33	1.33
			Convulsions	-	-	0.00	0.00
			Salivation	-	-	0.00	0.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	1.25	1.25	1.25
			Prostration	0.12	1.33	1.22	1.22
			Death	1.33	-	-	-
ATR/2-PAM/DZM	5.8	7CK	Appears Normal	168.00	240.00	0.00	0.00
			Tremors	0.00	0.75	0.75	0.75
			Convulsions	-	-	0.00	0.00
			Salivation	-	-	0.00	0.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	168.00	2.00	6.00
			Prostration	-	-	0.00	0.00
			Death	-	-	-	-
ATR/2-PAM/DZM	6.6	71G	Appears Normal	168.00	240.00	0.00	0.00
			Tremors	0.75	1.75	0.75	0.75
			Convulsions	-	-	0.00	0.00
			Salivation	-	-	0.00	0.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	96.00	2.00	6.00
			Prostration	-	-	0.00	0.00
			Death	-	-	-	-
ATR/2-PAM/DZM	7.3	78V	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	12.00	2.00	6.00
			Convulsions	-	-	0.00	0.00
			Salivation	-	-	0.00	0.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	240.00	2.00	6.00
			Prostration	0.15	0.50	0.35	0.35
			Death	-	-	-	-
ATR/2-PAM/DZM	10.9	6S4	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	1.25	1.25	1.25
			Convulsions	0.12	0.50	0.38	0.38
			Salivation	-	-	0.00	0.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	1.27	1.27	1.27
			Prostration	0.12	1.27	1.15	1.15
			Death	1.27	-	-	-

TABLE D-21.
(Continued)

Treatment Group	Calculated GD Dose ^(a) ($\mu\text{g/kg}$)	Animal	Clinical Sign	Time to onset (hr)	Time to last obs. (hr)	Duration of sign in 1st 2 hr (hrs)	Duration of sign in 1st 6 hr (hrs)
ATR/2-PAM/DZM	11.6	75U	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	0.75	0.75	0.75
			Convulsions	-	-	0.00	0.00
			Salivation	-	-	0.00	0.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	0.83	0.83	0.83
			Prostration	0.10	0.83	0.73	0.73
			Death	0.83	-	-	-
ATR/2-PAM/DZM	12.3	7AU	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	0.70	0.45	0.45
			Convulsions	0.10	0.50	0.40	0.40
			Salivation	-	-	0.00	0.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	0.70	0.70	0.70
			Prostration	0.07	0.70	0.63	0.63
			Death	0.70	-	-	-
ATR/2-PAM/DZM	15.3	6W6	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	0.42	0.42	0.42
			Convulsions	-	-	0.00	0.00
			Salivation	0.25	0.42	0.17	0.17
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	0.42	0.42	0.42
			Prostration	0.08	0.42	0.33	0.33
			Death	0.42	-	-	-
ATR/2-PAM/DZM	17.9	7CC	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	12.00	2.00	6.00
			Convulsions	-	-	0.00	0.00
			Salivation	6.00	70.67	0.00	0.00
			Miosis	18.00	70.67	0.00	0.00
			Mydriasis	0.00	12.00	2.00	6.00
			Prostration	0.07	70.67	1.93	5.93
			Death	70.67	-	-	-
ATR/2-PAM/DZM	19.4	H453	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	2.50	2.00	2.50
			Convulsions	-	-	0.00	0.00
			Salivation	-	-	0.00	0.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	2.83	2.00	2.83
			Prostration	0.03	2.83	1.97	2.80
			Death	2.83	-	-	-
ATR/2-PAM/DZM	28.5	H264	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	1.25	1.25	1.25
			Convulsions	-	-	0.00	0.00
			Salivation	0.00	0.50	0.50	0.50
			Miosis	1.00	1.50	0.50	0.50
			Mydriasis	0.00	1.00	1.00	1.00
			Prostration	0.03	1.50	1.47	1.47
			Death	1.50	-	-	-

TABLE D-21.
(Continued)

Treatment Group	Calculated GD Dose ^(a) (µg/kg)	Animal	Clinical Sign	Time to onset (hr)	Time to last obs. (hr)	Duration of sign in 1st 2 hr (hrs)	Duration of sign in 1st 6 hr (hrs)
PYR/ATR/2-PAM	79.4	H398	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	12.00	2.00	6.00
			Convulsions	-	-	0.00	0.00
			Salivation	0.00	24.00	0.75	1.75
			Miosis	0.00	48.00	0.25	3.75
			Mydriasis	0.25	2.50	1.75	2.25
			Prostration	0.02	72.00	1.98	5.98
			Death	-	-	-	-
PYR/ATR/2-PAM	129.4	6WG	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	5.00	2.00	4.00
			Convulsions	-	-	0.00	0.00
			Salivation	0.00	48.00	1.00	3.00
			Miosis	0.00	96.00	2.00	6.00
			Mydriasis	0.00	0.25	0.25	0.25
			Prostration	0.05	96.00	1.95	5.95
			Death	-	-	-	-
PYR/ATR/2-PAM	158.3	6TY	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	96.00	2.00	6.00
			Convulsions	-	-	0.00	0.00
			Salivation	0.00	12.00	1.00	2.50
			Miosis	0.00	12.00	1.25	5.25
			Mydriasis	0.00	1.50	1.00	1.00
			Prostration	0.02	144.00	1.98	5.98
			Death	-	-	-	-
PYR/ATR/2-PAM	173.8	73C	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	12.00	1.75	5.75
			Convulsions	-	-	0.00	0.00
			Salivation	0.00	24.00	0.75	2.25
			Miosis	0.00	72.00	2.00	6.00
			Mydriasis	0.00	0.25	0.25	0.25
			Prostration	0.03	72.00	1.97	5.97
			Death	-	-	-	-
PYR/ATR/2-PAM	209.4	75Z	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	12.00	2.00	6.00
			Convulsions	0.35	0.75	0.40	0.40
			Salivation	0.00	72.00	2.00	5.00
			Miosis	0.00	48.00	2.00	6.00
			Mydriasis	0.00	0.25	0.25	0.25
			Prostration	0.03	72.00	1.97	5.97
			Death	-	-	-	-
PYR/ATR/2-PAM	212.3	H525	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	0.08	0.08	0.08
			Convulsions	-	-	0.00	0.00
			Salivation	-	-	0.00	0.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	0.08	0.08	0.08
			Prostration	0.05	0.08	0.03	0.03
			Death	0.08	-	-	-

TABLE D-21.
(Continued)

Treatment Group	Calculated GD Dose ^(a) ($\mu\text{g/kg}$)	Animal	Clinical Sign	Time to onset (hr)	Time to last obs. (hr)	Duration of sign in 1st 2 hr (hrs)	Duration of sign in 1st 6 hr (hrs)
PYR/ATR/2-PAM	199.7	6T4	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	0.08	0.08	0.08
			Convulsions	-	-	0.00	0.00
			Salivation	0.00	0.08	0.08	0.08
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	0.08	0.08	0.08
			Prostration	0.03	0.08	0.05	0.05
			Death	0.08	-	-	-
PYR/ATR/2-PAM	208.3	73P	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	0.67	0.67	0.67
			Convulsions	-	-	0.00	0.00
			Salivation	0.00	0.50	0.50	0.50
			Miosis	0.00	0.67	0.67	0.67
			Mydriasis	0.00	0.25	0.25	0.25
			Prostration	0.03	0.67	0.63	0.63
			Death	0.67	-	-	-
PYR/ATR/2-PAM	208.4	H074	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	2.50	2.00	2.50
			Convulsions	-	-	0.00	0.00
			Salivation	0.00	47.92	1.50	5.50
			Miosis	0.00	12.00	1.00	5.00
			Mydriasis	0.00	0.75	0.75	0.75
			Prostration	0.03	47.92	1.97	5.97
			Death	47.92	-	-	-
PYR/ATR/2-PAM	257.8	H585	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	1.00	1.00	1.00
			Convulsions	-	-	0.00	0.00
			Salivation	0.00	30.58	0.25	4.25
			Miosis	0.00	30.58	2.00	6.00
			Mydriasis	0.00	0.50	0.50	0.50
			Prostration	0.03	30.58	1.97	5.97
			Death	30.58	-	-	-
PYR/ATR/2-PAM/DZM	60.3	79P	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	12.00	0.25	0.75
			Convulsions	0.13	0.25	0.12	0.12
			Salivation	2.50	4.00	0.00	1.50
			Miosis	0.25	240.00	1.25	5.25
			Mydriasis	0.00	0.25	0.25	0.25
			Prostration	0.03	12.00	1.97	5.97
			Death	-	-	-	-
PYR/ATR/2-PAM/DZM	76.1	H472	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	0.73	0.73	0.73
			Convulsions	-	-	0.00	0.00
			Salivation	-	-	0.00	0.00
			Miosis	0.00	0.73	0.73	0.73
			Mydriasis	0.00	0.25	0.25	0.25
			Prostration	0.03	0.73	0.70	0.70
			Death	0.73	-	-	-

TABLE D-21.
(Continued)

Treatment Group	Calculated GD Dose ^(a) ($\mu\text{g/kg}$)	Animal	Clinical Sign	Time to onset (hr)	Time to last obs. (hr)	Duration of sign in 1st 2 hr (hrs)	Duration of sign in 1st 6 hr (hrs)
PYR/ATR/2-PAM/DZM	75.4	7C6	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	12.00	2.00	6.00
			Convulsions	-	-	0.00	0.00
			Salivation	0.00	72.00	1.00	1.00
			Miosis	0.25	72.00	1.75	5.75
			Mydriasis	0.00	0.25	0.25	0.25
			Prostration	0.03	72.00	1.97	4.47
			Death	-	-	-	-
PYR/ATR/2-PAM/DZM	78.1	5R2	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	164.58	2.00	6.00
			Convulsions	-	-	0.00	0.00
			Salivation	0.00	72.00	1.50	2.50
			Miosis	3.00	48.00	0.00	3.00
			Mydriasis	0.00	0.25	0.25	0.25
			Prostration	0.03	164.58	1.97	5.97
			Death	164.58	-	-	-
PYR/ATR/2-PAM/DZM	96.4	7C4	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	47.67	2.00	6.00
			Convulsions	-	-	0.00	0.00
			Salivation	0.00	47.67	1.75	2.75
			Miosis	0.00	47.67	0.75	4.25
			Mydriasis	0.00	0.25	0.25	0.25
			Prostration	0.03	47.67	1.97	5.97
			Death	47.67	-	-	-
PYR/ATR/2-PAM/DZM	108.8	H309	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	0.73	0.73	0.73
			Convulsions	0.28	0.50	0.22	0.22
			Salivation	0.00	0.73	0.73	0.73
			Miosis	0.00	0.73	0.73	0.73
			Mydriasis	0.00	0.25	0.25	0.25
			Prostration	0.02	0.73	0.72	0.72
			Death	0.73	-	-	-
PYR/ATR/2-PAM/DZM	127.9	H300	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	0.75	0.75	0.75
			Convulsions	-	-	0.00	0.00
			Salivation	0.00	0.25	0.25	0.25
			Miosis	0.00	1.73	1.73	1.73
			Mydriasis	0.00	0.25	0.25	0.25
			Prostration	0.03	1.73	1.70	1.70
			Death	1.73	-	-	-
PYR/ATR/2-PAM/DZM	158.6	7D6	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	144.00	2.00	6.00
			Convulsions	-	-	0.00	0.00
			Salivation	0.00	96.00	1.00	2.00
			Miosis	0.00	72.00	2.00	6.00
			Mydriasis	0.00	0.25	0.25	0.25
			Prostration	0.02	144.00	1.98	5.98
			Death	-	-	-	-

TABLE D-21.
(Continued)

Treatment Group	Calculated GD Dose ^(a) ($\mu\text{g/kg}$)	Animal	Clinical Sign	Time to onset (hr)	Time to last obs. (hr)	Duration of sign in 1st 2 hr (hrs)	Duration of sign in 1st 6 hr (hrs)
PYR/ATR/2-PAM/DZM	198.7	H612	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	0.75	0.75	0.75
			Convulsions	0.18	0.75	0.57	0.57
			Salivation	0.00	0.75	0.75	0.75
			Miosis	0.00	0.50	0.50	0.50
			Mydriasis	0.00	0.75	0.50	0.50
			Prostration	0.03	0.75	0.72	0.72
			Death	0.75	-	-	-
PYR/ATR/2-PAM/DZM	258.9	7AL	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	5.50	2.00	3.00
			Convulsions	-	-	0.00	0.00
			Salivation	0.00	12.00	2.00	5.50
			Miosis	0.00	12.00	2.00	6.00
			Mydriasis	0.00	0.25	0.25	0.25
			Prostration	0.03	12.00	1.97	5.97
			Death	20.75	-	-	-
ATR/2-PAM (Cage)	20.1	5WT	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	1.25	1.25	1.25
			Convulsions	-	-	0.00	0.00
			Salivation	-	-	0.00	0.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	1.47	1.47	1.47
			Prostration	0.07	1.47	1.40	1.40
			Death	1.47	-	-	-
ATR/2-PAM (Cage)	20.6	I438	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	24.00	2.00	5.50
			Convulsions	-	-	0.00	0.00
			Salivation	0.25	27.68	0.25	0.75
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	27.68	2.00	6.00
			Prostration	0.12	27.68	1.88	5.88
			Death	27.68	-	-	-
ATR/2-PAM (Cage)	20.7	6W8	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	48.00	2.00	5.00
			Convulsions	-	-	0.00	0.00
			Salivation	-	-	0.00	0.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	72.00	2.00	6.00
			Prostration	0.17	72.00	1.83	5.33
			Death	-	-	-	-
ATR/2-PAM (Cage)	20.6	74H	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	0.50	0.50	0.50
			Convulsions	-	-	0.00	0.00
			Salivation	-	-	0.00	0.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	0.75	0.75	0.75
			Prostration	0.10	0.75	0.65	0.65
			Death	0.75	-	-	-

TABLE D-21.
(Continued)

Treatment Group	Calculated GD Dose ^(a) (μ g/kg)	Animal	Clinical Sign	Time to onset (hr)	Time to last obs. (hr)	Duration of sign in 1st 2 hr (hrs)	Duration of sign in 1st 6 hr (hrs)
ATR/2-PAM (Cage)	20.6	7C9	Appears Normal	-	-	0.00	0.00
			Tremors	0.00	1.72	1.72	1.72
			Convulsions	-	-	0.00	0.00
			Salivation	-	-	0.00	0.00
			Miosis	-	-	0.00	0.00
			Mydriasis	0.00	1.72	1.72	1.72
			Prostration	0.08	1.72	1.63	1.63
			Death	1.72	-	-	-

^(a) GD doses calculated from weight losses of syringes and chemical analysis of dosing solution.

^(b) Sign was not noted during duration of the experiment.

^(c) Excessive salivation or bronchial secretions.

TABLE D-22. DESCRIPTIVE STATISTICS OF CLINICAL SIGNS FOR
FOUR TREATMENT GROUPS IN PHASE V EXPERIMENTS
BASED ON NONMISSING UNCENSORED ENDPOINTS

Clinical Sign	Endpoint	Treatment Group											
		ATR/2-PAM			ATR/2-PAM/DZM			PYR/ATR/2-PAM			PYR/ATR/2-PAM/DZM		
		N ^(a)	Mean	(S.D.)	N	Mean	(S.D.)	N	Mean	(S.D.)	N	Mean	(S.D.)
			(hr)			(hr)			(hr)			(hr)	
Tremors	Time to onset	7	0.00	(0.00)	10	0.08	(0.24)	10	0.00	(0.00)	10	0.00	(0.00)
	Duration in 1st 2 hrs	8	1.20	(0.75)	10	1.16	(0.64)	10	1.36	(0.82)	10	1.32	(0.73)
	Duration in 1st 6 hrs	8	2.14	(2.06)	10	2.01	(2.19)	10	3.21	(2.62)	10	3.07	(2.61)
Convulsions	Time to onset	2	0.15	(0.07)	2	0.11	(0.01)	1	0.35	(-)	3	0.20	(0.08)
	Duration in 1st 2 hrs	8	0.12	(0.22)	10	0.08	(0.17)	10	0.04	(0.13)	10	0.09	(0.18)
	Duration in 1st 6 hrs	8	0.12	(0.22)	10	0.08	(0.17)	10	0.04	(0.13)	10	0.09	(0.18)
Salivation ^(b)	Time to onset	0	-	(-)	3	2.08	(3.39)	9	0.00	(0.00)	9	0.28	(0.83)
	Duration in 1st 2 hrs	8	0.00	(0.00)	10	0.07	(0.16)	10	0.78	(0.63)	10	0.90	(0.70)
	Duration in 1st 6 hrs	8	0.00	(0.00)	10	0.07	(0.16)	10	2.48	(1.98)	10	1.70	(1.62)
Miosis	Time to onset	0	-	(-)	2	9.50	(12.02)	8	0.00	(0.00)	10	0.35	(0.94)
	Duration in 1st 2 hrs	8	0.00	(0.00)	10	0.05	(0.16)	10	1.12	(0.86)	10	1.14	(0.70)
	Duration in 1st 6 hrs	8	0.00	(0.00)	10	0.05	(0.16)	10	3.87	(2.62)	10	3.40	(2.33)
Mydriasis	Time to onset	8	0.03	(0.09)	10	0.00	(0.00)	10	0.03	(0.08)	10	0.00	(0.00)
	Duration in 1st 2 hrs	8	1.38	(0.63)	10	1.42	(0.65)	10	0.52	(0.52)	10	0.28	(0.08)
	Duration in 1st 6 hrs	8	3.38	(2.74)	10	3.10	(2.57)	10	0.57	(0.66)	10	0.28	(0.08)
Prostration	Time to onset	5	0.15	(0.04)	8	0.08	(0.04)	10	0.03	(0.01)	10	0.03	(0.01)
	Duration in 1st 2 hrs	8	0.52	(0.48)	10	0.86	(0.74)	10	1.45	(0.85)	10	1.56	(0.60)
	Duration in 1st 6 hrs	8	0.52	(0.48)	10	1.34	(1.82)	10	4.25	(2.77)	10	3.82	(2.52)
Death	Time to death ^(c)	4	0.91	(0.29)	6	1.26	(0.86)	5	15.87	(22.21)	6	12.06	(19.15)

^(a) For times to onset and death, N is the number of animals that responded. For durations, N is the number of animals in study group.

^(b) Excessive salivation or bronchial secretions.

^(c) Based on 48-hr endpoint.

TABLE D-23. SUMMARY OF STATISTICAL COMPARISONS BETWEEN CLINICAL SIGNS FOR FOUR TREATMENT GROUPS FROM PHASE V EXPERIMENTS

Clinical Sign	Incidence of Clinical Sign				Fisher's Exact Test	Endpoint	Wilcoxon Test p-value	Analysis of Variance Results Using Censored Values								Chi-Square p-value	Log-Dose Slope p-value
	ATR/2-PAM	ATR/2-PAM/DZM	PYR/ATR/2-PAM	PYR/ATR/2-PAM/DZM				2-PAM		2-PAM/DZM		PYR/2-PAM		PYR/2-PAM/DZM			
	7/8	10/10	10/10	10/10				Predicted	Mean (S.E.)	Predicted	Mean (S.E.)	Predicted	Mean (S.E.)	Predicted	Mean (S.E.)		
Tremors					0.474	Time, 1st onset	-	0.04 (0.02)	0.02 (0.01)	0.01 (0.00)	0.01 (0.01)	0.01 (0.01)	0.460	0.072			
Convulsions						Duration, 2 hrs	0.605	1.64 (0.26)	1.36 (0.20)	1.83 (0.22)	1.61 (0.21)	0.478	-				
						Duration, 6 hrs	0.308	3.72 (0.91)	2.55 (0.69)	4.51 (0.75)	4.16 (0.74)	0.228	-				
	2/8	2/10	1/10	3/10	1.000	Time, 1st onset	-	9.75 (10.0)	6.81 (6.29)	8.78 (8.06)	10.21 (9.57)	0.090	0.011*				
						Duration, 2 hrs	0.806	0.12 (0.06)	0.08 (0.05)	0.04 (0.05)	0.10 (0.05)	0.789	-				
Salivation/ Bronchial Secretions						Duration, 6 hrs	0.806	0.12 (0.06)	0.08 (0.05)	0.04 (0.05)	0.10 (0.05)	0.789	-				
	0/8	3/10	9/10	9/10	0.000*	Time, 1st onset	-	15.09 (9.70)	5.00 (2.89)	0.01 (0.01)	0.05 (0.03)	0.261	0.000*				
						Duration, 2 hrs	0.000*	0.00 (0.17)	0.10 (0.15)	0.87 (0.15)	0.99 (0.16)	0.000*	-				
						Duration, 6 hrs	0.000*	0.00 (0.43)	0.16 (0.39)	2.75 (0.40)	2.02 (0.41)	0.000*	-				
Miosis	0/8	2/10	8/10	10/10	0.000*	Time, 1st onset	-	14.97 (9.44)	8.73 (4.95)	0.01 (0.01)	0.05 (0.03)	0.859	0.000*				
Mydriasis						Duration, 2 hrs	0.000*	0.00 (0.20)	0.08 (0.18)	1.19 (0.19)	1.33 (0.20)	0.000*	-				
						Duration, 6 hrs	0.000*	0.00 (0.53)	0.16 (0.48)	4.23 (0.50)	4.42 (0.56)	0.000*	-				
	8/8	10/10	10/10	10/10	1.000	Time, 1st onset	-	0.02 (0.00)	0.01 (0.00)	0.01 (0.00)	0.01 (0.00)	0.159	0.100				
						Duration, 2 hrs	0.000*	1.80 (0.15)	1.84 (0.14)	0.63 (0.12)	0.30 (0.11)	0.000*	-				
Prostration						Duration, 6 hrs	0.000*	5.00 (0.55)	5.02 (0.54)	0.83 (0.41)	0.37 (0.39)	0.000*	-				
	5/8	8/10	10/10	10/10	0.017*	Time, 1st onset	-	3.60 (3.09)	0.58 (0.44)	0.03 (0.02)	0.05 (0.04)	0.108	0.001*				
						Duration, 2 hrs	0.001*	0.73 (0.21)	1.15 (0.20)	1.97 (0.20)	2.02 (0.21)	0.000*	-				
						Duration, 6 hrs	0.000*	1.06 (0.49)	2.27 (0.49)	5.97 (0.46)	5.72 (0.49)	0.000*	-				
Death	4/8	6/10	5/10	6/10	1.000	Time to death	-	25.1 (31.8)	22.6 (26.3)	29.6 (34.0)	62.3 (74.0)	0.030*	0.003*				

Explanation of column headings

Incidence of clinical sign: proportion of animals in each group that exhibited the clinical sign.

Fisher's Exact Test: p-value for test comparing the incidence of clinical signs between non-PYR pretreated animals (ATR/2-PAM and ATR/2-PAM/DZM) and PYR pretreated animals (PYR/ATR/2-PAM and PYR/ATR/2-PAM/DZM).

Wilcoxon Test: p-value for Wilcoxon's rank sum test comparing nonmissing, uncensored durations between non-PYR pretreated animals (ATR/2-PAM and ATR/2-PAM/DZM) and PYR pretreated animals (PYR/ATR/2-PAM and PYR/ATR/2-PAM/DZM).

Analyses of Variance Using Censored Values: (PYR/ATR/2-PAM and PYR/ATR/2-PAM/DZM). This test was not performed on times to onset since they were determined to be dose-dependent.

Predicted Mean: The mean value of endpoint predicted by the ANOVA for each group. S.E. is the standard error of the predicted mean. Predicted values were computed at the GD 48 hr LD₅₀ for each group.

Chi-square p-value: p-value for comparing the mean predicted values among all four treatment groups. 8.81 µg/kg for ATR/2-PAM, 11.1 µg/kg for ATR/2-PAM/DZM, 182 µg/kg for PYR/ATR/2-PAM, and 94.5 µg/kg for PYR/ATR/2-PAM/DZM.

Log-dose Slope p-value: p-value for the log GD dose slope. P-values for the log-dose slope were not significant for durations within 2 hr and 6 hr, so the log GD dose covariate was dropped from the model for duration.

* Statistically significant at the 0.05 level.